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(54) Title: CHARACTERIZATION OF *MYCOPLASMA HYOPNEUMONIAE* ADHESINS

(57) Abstract

The present invention provides for the identification, purification and characterization of *Mycoplasma hyopneumoniae* adhesins. The *Mycoplasma hyopneumoniae* adhesins of the invention were purified using an affinity chromatography procedure which incorporates a novel *Mycoplasma hyopneumoniae* receptor analogue for the attachment and/or removal of the adhesins from a mycoplasmal preparation. In another aspect of the invention, there is provided an *in vitro* microtiter plate adherence assay for the characterization of *Mycoplasma hyopneumoniae* and adhesins thereof. In addition, the effect of passage level on adherence of *Mycoplasma hyopneumoniae* was evaluated; the selection of high-adherent and low-adherent clones conducted; and monoclonal antibodies against the adhesins were raised and used to study adherence inhibition.

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CHARACTERIZATION OF MYCOPLASMA HYOPNEUMONIAE ADHESINS

This invention was made with partial Government support under USDA Grant No. 91-37204-6488 and the Iowa Livestock Health Advisory Council. The Government has certain rights in this invention.

FIELD OF THE INVENTION

The present invention relates generally to the organism *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*), the pathogen of swine mycoplasmal pneumonia. More particularly, the invention relates to the identification, purification and characterization of *M. hyopneumoniae* adhesins, and their use in vaccines and diagnostic testing.

BACKGROUND OF THE INVENTION

Mycoplasma hyopneumoniae (M. hyopneumoniae), the pathogen of swine mycoplasmal pneumonia, is an economically important and widely spread swine disease. This organism has a strict tropism for ciliated cells in the respiratory epithelium of pigs. Moreover, attachment of this organism to ciliated cells is considered an important event for development of chronic pneumonia in pigs. Once infected with *M. hyopneumoniae*, the pig is predisposed to infections by other bacteria and viruses because infection by *M. hyopneumoniae* causes extensive loss of cilia, which compromises the muco-ciliary clearance system of the pig.

Adherence to swine ciliated cells is generally considered a prerequisite for colonization and development of lung lesions. Therefore, it is postulated that vaccines with improved efficacy could be developed based on adherence mechanisms. However, the mechanism of adherence (attachment) of *M. hyopneumoniae* is a rather complicated event, not fully understood today. Adherence is believed to include ligand-receptor mediated interactions as well as hydrophobic interactions. Although vaccines for *M. hyopneumoniae* are commercially available, they only partially reduce development of lung lesions and do not protect pigs from infection.

Several *in vitro* adherence models have been established for use in characterizing the adherence mechanisms of mycoplasma. For example, in Young et al., Hemagglutination and Hemagglutination Inhibition of Turkey Red Blood Cells with *Mycoplasma hyopneumoniae*, 1989, *Am. J. Vet. Res.*, vol. 50, pp. 1052-1055, which is hereby incorporated by reference, the authors evaluated the hemagglutination of turkey RBC by *M. hyopneumoniae* and the inhibitory effect of antibodies on hemagglutination to develop an *in vitro* cytadsorption assay. The authors determined that *M. hyopneumoniae* convalescent porcine serum and monoclonal antibodies against two *M. hyopneumoniae* immunogens of molecular weights 64,000 and 41,000 inhibited hemagglutination.

In Zielinski et al., Adherence of *Mycoplasma hyopneumoniae* to Cell Monolayers, March 1990, *Am. J. Vet. Res.*, vol. 51, No. 3, pp. 339-343, which is hereby incorporated by reference, the authors describe an *in vitro* adherence model for *M. hyopneumoniae*, using monolayers of human and porcine lung fibroblasts and porcine kidney cells. *M. hyopneumoniae* grown in Friis mycoplasmal broth was radiolabeled, washed, concentrated and inoculated on the monolayers. After centrifugation to facilitate adherence, the monolayers were washed, dissolved with NaOH and suspended in scintillation liquid. Thereafter, the radioactivity was determined in a liquid scintillation counter. In a related paper, Zielinski et al., Effect of Growth in Cell Cultures and Strain on Virulence of *Mycoplasma hyopneumoniae* for Swine, March 1990, *Am. J. Vet. Res.*, vol. 51, No. 3, pp. 344-348, which is hereby incorporated by reference, the authors studied whether growth of *M. hyopneumoniae* in a cell monolayer would enhance its virulence; and compared the virulence of various strains of the organism. The authors concluded that *M. hyopneumoniae* induced pneumonia when inoculated into pigs, regardless of whether it was grown in a regular mycoplasmal broth or co-cultured with human lung fibroblasts.

In Young et al., Analysis of Virulence-Associated Antigens of *Mycoplasma hyopneumoniae*, 1992, IOM Letters, 2:321, which disclosure is hereby incorporated by reference, from the Abstract, the authors identified antigens associated with virulence of the pathogenic strains of mycoplasma and the effects of *in vitro* passages.

In Zhang et al., Characterization of Receptors for *Mycoplasma hyopneumoniae* Adherence to Swine Respiratory Epithelium, 1992, *Proc. Am. Soc. Micro.*, 92:164, which disclosure is hereby incorporated by reference, from the Abstract, the authors identified receptor analogues for *Mycoplasma hyopneumoniae* attachment by using a microtiter plate

assay. The assay comprised first extracting cilia from tracheal epithelial cells of SPF pigs and immobilizing them onto ELISA plates after treatment with SDS. Then, *M. hyopneumoniae* and its antibodies conjugated with enzymes were sequentially added to detect binding of the organism to immobilized cilia extract. The authors determined that receptor analogues including heparin, mucin, fucoidin, and chondroitin sulfate interacted with surface molecules of mycoplasmas and blocked adherence in the microtiter plate adherence assay.

In Zhang et al., Glycolipid Receptors for *Mycoplasma hyopneumoniae* Attachment, 1992, IOM Letters, 2:320, which disclosure is hereby incorporated by reference, from the Abstract, the authors identified three ciliary glycolipid receptors of the mycoplasma by thin layer chromatography. Here, various glycolipids were separated on a thin layer chromatography plate, which was then overlayed sequentially with mycoplasma cells, anti-mycoplasma antibodies and secondary antibody conjugates. Binding of mycoplasmas to specific glycolipids was visualized by treatment of the plate with 4-chloro-1-naphthol substrate.

In Zielinski et al., Adherence of *Mycoplasma hyopneumoniae* to Porcine Ciliated Respiratory Tract Cells, August 1993, *Am. J. Vet. Res.*, vol. 54, No. 8, which disclosure is hereby incorporated by reference, the authors describe an adherence assay comprising suspensions of porcine respiratory tract ciliated epithelial cells and *M. hyopneumoniae*. Here, epithelial cells, collected by use of cytologic brushes, were mixed with broth cultures of *M. hyopneumoniae* and the mixtures were incubated, diluted, vortexed and sedimented. Pellets were spread on glass slides, stained with a fluorescent antibody against *M. hyopneumoniae* and evaluated by fluorescent microscopy. The authors concluded that adherence of *M. hyopneumoniae* may be a host-specific event that is mediated by proteins and carbohydrates on the surface of the organism and by sulfur-containing molecules in the host cell membrane.

Although *in vitro* adherence models have been established for use in studying the adherence mechanisms of mycoplasma, to date, adhesins of *M. hyopneumoniae* have not been identified. Therefore, the need remains for the identification, purification and characterization of *M. hyopneumoniae* adhesins and host cell factors involved therewith.

SUMMARY OF THE INVENTION

Accordingly, the primary object of the present invention is to provide for the identification, purification and characterization of *M. hyopneumoniae* adhesins.

Another object of the present invention is to provide an *in vitro* model for such identification, purification and characterization.

The present invention provides for the identification and purification of novel adhesins of *M. hyopneumoniae*. As used herein, the term "adhesin(s)" refer(s) to *a M. hyopneumoniae* protein(s) which is involved in the adherence of *M. hyopneumoniae* to a host cell(s) and/or the virulence of the mycoplasma. As used herein, the term "protein(s)" refers to any of the following: a protein, polypeptide, glycoprotein and/or lipoprotein. The adhesins of the present invention were purified using affinity chromatography. The affinity chromatography technique utilized a novel *M. hyopneumoniae* receptor analogue for the attachment and/or removal of the adhesins from a mycoplasmal preparation.

In another aspect of the invention, there is provided a novel *in vitro* adherence assay for the characterization of *M. hyopneumoniae* adhesins as well as various strains of *M. hyopneumoniae*. More particularly: (1) *M. hyopneumoniae* strains LI27, FA1, 2A3 and J were compared for their adherence capability in the *in vitro* adherence assay of the invention; (2) the effect of passage level on adherence of *M. hyopneumoniae* was evaluated in the *in vitro* adherence assay of the invention; (3) the selection of high-adherent and low-adherent clones was conducted using the *in vitro* adherence assay of the invention; and (4) monoclonal antibodies against the adhesins were raised and used in the *in vitro* adherence assay of the invention to study adherence inhibition.

The adhesin(s) (i.e., proteins and/or polypeptides) of the present invention, or a portion thereof, can be combined with a pharmaceutically-acceptable carrier to form a vaccine or used alone for administration to porcine for preventing infection by *M. hyopneumoniae*. Alternatively, the adhesin(s) of the present invention can be used to raise an antibody or a binding fragment or portion thereof. The antibody or binding fragment or portion thereof may be used alone or combined with a pharmaceutically-acceptable carrier to treat swine already exposed to *M. hyopneumoniae* to induce passive immunity to prevent disease occurrence.

The adhesin(s) (i.e., proteins and/or polypeptides) of the present invention or the antibodies or binding fragments or portions thereof raised against the adhesin(s) can also be utilized in a method for detection of *M. hyopneumoniae* in a sample of tissue or fluids. For example, when the adhesin(s) is utilized, it can be provided as an antigen. Any reaction with the antigen or the antibody is detected using any conventional assay system, which then indicates the presence of *M. hyopneumoniae* in the sample.

Isolation of the adhesin(s) of the present invention constitutes a significant advance in the treatment and detection of *M. hyopneumoniae*. It also provides the basis for a vaccine to prevent infection by *M. hyopneumoniae* as well as an antisera for passive immunization for those porcine at risk of being exposed to *M. hyopneumoniae*. It is understood that once the adhesin(s) or antigenic or immunogenic portion thereof is sequenced, it can be produced at high levels for use in the vaccine by recombinant DNA technology.

In diagnostic applications, the adhesin(s) of the present invention as well as antibodies and binding fragments or portions thereof against the adhesin(s) can permit rapid determination of whether a particular porcine is infected with *M. hyopneumoniae*.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a graph showing differences in adherence of *M. hyopneumoniae* strains LI27, 2A3, J and FA1. (Bars represent mean OD±SD).

Fig. 2 is a graph showing the effect of passage level on adherence (Mycoplasmal proteins of different passages were adjusted to 11.25 and 22.5 μg/ml. Data represent mean OD±SD in three independent experiments and asterisk (*) indicates the passages at which significant reduction in adherence was observed compared to passage 3($P<0.01$)).

Fig. 3 is a graph showing fractions of mycoplasmal proteins (adhesins) separated by affinity chromatography and detected by a UV monitor at 280 nm.

Fig. 4 is a graph showing adherence activity of the fractions of Fig. 3.

Fig. 5 is a photograph of an SDS-polyacrylamide gel and immunoblot showing protein (adhesin) profiles in different fractions.

Fig. 6 is a graph showing dose-dependent binding of mycoplasmas to immobilized cilia in the microtiter plate adherence assay of the invention (data represent mean OD±SD in three independent experiments).

Fig. 7 is a bar graph showing effect of treatment of cilia with neuraminidase and sodium metaperiodate on adherence (Cilia were treated with sodium acetate buffer (A); neuraminidase (B); 0.01 M sodium metaperiodate (C); or 0.1 M sodium metaperiodate (D). Bars represent mean OD±SD in triplicate assays. Bars with different numbers were significantly different as determined by analysis of variance with the Tukey contrast at a significance level of 0.05.

Fig. 8 is a photograph showing mycoplasmal proteins of strain LI27 separated by SDS-PAGE and immunoblot and reacted with monoclonal antibodies F2G5 (Lane 1), F1B6 (Lane 2) and R6C10 (Lane 3). Sizes of proteins (in kilodaltons) are shown on the left.

Fig. 9 is a photograph showing *M. hyopneumoniae* (LI27) cells treated with graded doses of trypsin: 0.0 μg/ml (Lane 1), 0.01 μg/ml (Lane 2), 0.04 μg/ml (Lane 3), 0.1 μg/ml (Lane 4), 0.25 μg/ml (Lane 5), 0.5 μg/ml (Lane 6), 1 μg/ml (Lane 7), 0.5 μg/ml (Lane 8). A, SDS-PAGE profile stained with Coomassie brilliant blue. B, immunostaining with Mab F2G5. Sizes of proteins (in kilodaltons) are shown on the left of each panel.

Fig. 10 is a photograph showing digestion of *M. hyopneumoniae* (LI27) cells treated with various concentrations of carboxypeptidase Y: Lane 1, nontreated control; Lane 2, 0.2 μg/ml; Lane 3, 1.0 μg/ml; Lane 4, 5 μg/ml; Lane 5, 25 μg/ml; Lane 6, 125 μg/ml. Mycoplasmas were analyzed by SDS-PAGE and immunoblotted with Mab F2G5. Sizes of proteins (in kilodaltons) are shown on the left of each panel.

Fig. 11 is a photograph showing *M. hyopneumoniae* strains LI27, 2A3, J, 144L and FA1 immunoblotted with Mab F2G5.

Fig. 12 is a photograph of a transmission electron micrograph of mycoplasmas immunolabelled with Mab F2G5 and gold particles: A, mycoplasmas incubated with cell culture medium and gold-conjugates as a negative control. B, mycoplasmas reacted with Mab F2G5 and gold-conjugates. Arrows indicate the fuzzy structures labelled with gold particles. Bars represent 0.1 μm.

Fig. 13 is a photograph of an affinity chromatography gel showing purification of adhesins by Mab F2G5: Lane 1, total mycoplasmal proteins (LI27); Lane 2, elute from

Affi-Gel 10-Mab F2G5 column (1.5μg); Lane 3, 2 fold greater load of the eluate. A, SDS-PAGE profile stained with Coomassie brilliant blue. B, immunostaining with Mab F2G5. Sizes of proteins (in kilodaltons) are shown on the left and right of each panel.

Fig. 14 is a bar graph showing dose-dependent binding of adhesins purified by antibody affinity chromatography, diluted in adherence buffer and directly applied to wells of microtiter plates coated with solubilized cilia or with gelatin (negative control). Attached proteins were detected with hyperimmune antibodies. Data were expressed as mean OD ± SD of three independent experiments.

Fig. 15 is a photograph of F2G5-reacting size-variable antigens where mycoplasmal proteins (LI27) separated by SDS-PAGE were immunostained with Mab F2G5. The number of mycoplasmas (CCU) in each of the lanes were about 1 x 10^8 (Lane 1), 2.5 x 10^8 (Lane 2), 5 x 10^8 (Lane 3) and 1 x 10^9 (Lane 4). Sizes of proteins (in kilodaltons) are indicated on the left. The brackets indicate ladders of proteins recognized by Mab F2G5.

Fig. 16 is a photograph showing adhesins purified by antibody affinity chromatography digested with graded doses of trypsin (0.08, 0.2, 0.5 and 2.0 μg/ml). Samples were pooled, separated by SDS-PAGE and immunoblotted with Mab F2G5. Lane 1, mixture of digested adhesins (6 μg); Lane 2, nondigested adhesins (3 μg). Sizes of proteins (in kilodaltons) are indicated on the right.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides for the identification, purification and characterization of *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) and *M. hyopneumoniae* adhesins. In one aspect of the invention, there is provided a novel affinity chromatography technique for the identification and purification of *M. hyopneumoniae* adhesin(s). In another aspect of the invention, there is provided an *in vitro* adherence assay (microtiter plate adherence assay) for the characterization *of* *M. hyopneumoniae* and *M. hyopneumoniae* adhesins.

A modified affinity chromatography technique is utilized in the invention to facilitate isolation and purification of the adhesin(s). In this technique, a novel *M. hyopneumoniae* receptor analogue is chemically coupled to a substrate. The substrate

is a support comprising a material, such as, for example, paper, metal foils, polystyrene, polyesters [e.g., poly(ethylene terephthalate)], polycarbonates, cellulose esters (e.g., cellulose acetate), glass beads and agarose. The substrate, preferably agarose beads, are then placed in a commercially available small column. Thereafter, the adhesin(s) solution i.e., protein solution, is passed through the column. Only the adhesin(s) (protein(s)) to which the receptor analogue is directed will adhere to the column; all others will pass through unimpeded. The desired adhesin(s) can be eluted from the column by adding a solution that disrupts the binding to the receptor. Examples of receptor analogues within the scope of the invention include, without limitation, heparin, mucin, fucoidin, chondroitin sulfate and dextran sulfate. Heparin, being commercially available, is the preferred receptor analogue for use in the affinity chromatography technique of the invention.

More particularly, in one embodiment utilizing the modified affinity chromatography technique of the invention, mycoplasmas solubilized with CHAPS were fractionated by an affinity column packed with heparin-agarose beads. The void fraction was removed by washing with phosphate-buffered saline (PBS), while the proteins bound to heparin were eluted from the gel with 0.7M NaCl or a stepwise gradient of NaCl. It was determined (as described elsewhere herein) that the void fraction had much lower adherence activity than the fraction eluted from heparin-agarose gel in which adherence activity was enriched. SDS-PAGE and immunoblotting revealed that several proteins with estimated molecular weights 200 KDa (K), 185K, 145K, 116K, 100K, 97K, 57K and 43K were present in the fraction eluted from the affinity gel, but not in the void fraction.

Once the adhesin(s) of the invention is identified and purified as described above, the *in vitro* adherence assay of the invention is utilized to characterize the *M. hyopneumoniae* adhesin(s). In particular, the adherence assay of the invention was used to: (1) compare the adherence capability of *M. hyopneumoniae* strains LI27, FA1, 2A3 and J; (2) evaluate the effect of passage level on adherence of *M. hyopneumoniae*; (3) conduct the selection of high-adherent and low-adherent clones; and (4) study adherence inhibition using monoclonal antibodies raised against the adhesin(s).

The *in vitro* adherence assay of the invention is a microtiter plate adherence assay. *M. hyopneumoniae* strains LI27, FA1, 2A3 and J were used in the assay for purposes of illustration only. It is understood that *M. hyopneumoniae* adhesins isolated from any strain

of *M. hyopneumoniae* or clones thereof are within the scope of the present invention. For example, isolates from pigs inoculated with type strain 11 include 232 and LI27 and any clones from these two strains such as 232 clones 2A3 and FA1, LI27 clones 60 and 91 and other isolates from field isolates from Iowa State University, Ames, Iowa or isolates from pigs inoculated with a field isolate including 3-14, 4-14, 16-14, 18-14, 31-9, 37-9,144, 194, 1262, 1361, 1361A, 1363, 1375A, 1375C, 1378A, 1381A, 1416B, 1417, 1419, 1424B, 1464A, 1472C, 8489, 8689, 8765, 9078, 9275, 10731, 10954, 10986, 11684C, 11928, and any clones from these isolates. As used herein, the term "clone(s)" refers to a single colony which is derived and/or cultured from a homogeneous parent strain of *M. hyopneumoniae*.

In one embodiment thereof, the assay comprises first extracting cilia from tracheal epithelial cells of pigs and immobilizing them onto ELISA (microtiter) plates after treatment with SDS. Then, *M. hyopneumoniae* and its antibodies conjugated with enzymes are sequentially added to detect binding of the organism to immobilized cilia extract.

It was determined that two monoclonal antibodies (Mabs) against multiple mycoplasmal adhesins (proteins) produced dose-dependent adherence inhibition in the microtiter plate adherence assay. Pathogenic strain LI27 had significantly higher adherence activity than strain J which is unable to cause disease in pigs. It was further determined that increasing *in vitro* passage levels decreased adherence capability of *M. hyopneumoniae*, which is believed to be correlated with its virulence. Among 81 individual *M. hyopneumoniae* clones screened, it was found that most had intermediate adherence activity. Two clones were identified with substantially higher activity (clone 91) or lower adherence activity (clone 60), respectively. Preliminary analysis with immunoblotting revealed that a 145K protein disappeared in the low-adherent clone. It is believed that multiple adhesins are involved in adherence of *M. hyopneumoniae* to host cells, and several of such adhesins i.e., 145K and 97 KDa proteins are major adhesins. These findings provide a feasible approach to development of improved vaccines against mycoplasmal pneumonia in swine.

It is understood that using the adhesin(s) of the present invention that a wide array of therapeutic and/or prophylactic agents and diagnostic procedures for, respectively, treating and detecting *M. hyopneumoniae* can be developed. For example, an effective amount of the adhesin(s) i.e., protein or polypeptide, of the present invention can be

administered alone or in combination with a pharmaceutically-acceptable carrier to pigs, as a vaccine, for preventing infection by *M. hyopneumoniae*. Alternatively, it is possible to administer to pigs exposed to *M. hyopneumoniae* an effective amount of an antibody or binding fragment or portion thereof against the adhesin(s) as a passive immunization. Such antibodies or binding fragments or portions thereof can be administered alone or in combination with a pharmaceutically-acceptable carrier to effect short term treatment of pigs are at risk of being exposed to *M. hyopneumoniae*.

Antibodies suitable for use in inducing passive immunity can be monoclonal or polyclonal. Monoclonal antibody production may be effected by techniques which are well-known in the art. Basically, the process involves first obtaining immune cells (lymphocytes) from the spleen of a mammal (i.e., mouse) which has been previously immunized with the antigen of interest (i.e. adhesin (protein or polypeptide) of the present invention) either *in vivo* or *in vitro*. The antibody-secreting lymphocytes are then fused with (mouse) myeloma cells or transformed cells, which are capable of replicating indefinitely in cell culture, thereby producing an immortal, immunoglobulin-secreting cell line. The resulting fused cells, or hybridomas, are cultured and the resulting colonies screened for the production of the desired monoclonal antibodies. Colonies producing such antibodies are cloned, and grown either *in vivo* or *in vitro* to produce large quantities of antibody. A description of the theoretical basis and practical methodology of fusing such cells is set forth in Kohler and Milstein, *1975, Nature*, 256:495, which is hereby incorporated by reference.

Mammalian lymphocytes are immunized by *in vivo* immunization of the animal (i.e., a mouse) with the adhesin(s) i.e., antigen, of the present invention. Such immunizations are repeated as necessary at intervals of up to several weeks to obtain a sufficient titer of antibodies. Following the last antigen boost, the animals are sacrificed and spleen cells removed.

Fusion with mammalian myeloma cells or other fusion partners capable of replicating indefinitely in cell culture is effected by standard and well-known techniques, for example, by using polyethylene glycol (PEG) or other fusing agents. See Milstein and Kohler, 1976, *Eur. J. Immunol.*, 6:511, which is hereby incorporated by reference. This immortal cell line, which is preferably murine, but may also be derived from cells of other mammalian species, including but not limited to rats and humans, is selected to be

deficient in enzymes necessary for the utilization of certain nutrients, to be capable of rapid growth and to have good fusion capability. Many such cell lines are known to those skilled in the art, and others are regularly described.

Procedures for raising polyclonal antibodies are also well known. Typically, such antibodies can be raised by administering the adhesin(s) of the present invention subcutaneously to New Zealand white rabbits which have first been bled to obtain pre-immune serum. The antigens can be injected at a total volume of 100 µl per site at six different sites. Each injected material will contain synthetic surfactant adjuvant pluronic polyols, or pulverized acrylamide gel containing the protein or polypeptide after SDS-polyacrylamide gel electrophoresis. The rabbits are then bled two weeks after the first injection and periodically boosted with the same antigen three times every six weeks. A sample of serum is then collected 10 days after each boost. Ultimately, the rabbits are euthanatized with pentobarbital 150 mg/Kg IV. This and other procedures for raising polyclonal antibodies are disclosed in E. Harlow et. al., editors, *Antibodies: A Laboratory Manual*, 1988, which is hereby incorporated by reference.

The vaccines and passive immunization agents of this invention can be administered orally, parenterally such as, subcutaneously, intravenously, intramuscularly, by intranasal instillation, or by application to mucous membranes, such as, that of the nose, throat, and bronchial tubes. They may be administered alone or with suitable pharmaceutical carriers, and can be in solid or liquid form such as, tablets, capsules, powders, solutions, suspensions, or emulsions.

The solid unit dosage forms can be of the conventional type. The solid form can be a capsule, such as an ordinary gelatin type containing the adhesin(s) i.e., protein or polypeptide, of the present invention or the antibody or binding fragment or portion thereof raised against the adhesin(s) and a carrier, for example, lubricants and inert fillers such as, lactose, sucrose, or cornstarch. In another embodiment, these compounds are tableted with conventional tablet bases such as lactose, sucrose, or cornstarch in combination with binders like acacia, cornstarch, or gelatin, disintegrating agents such as, cornstarch, potato starch, or alginic acid, and a lubricant like stearic acid or magnesium stearate.

The adhesin(s) i.e., protein or polypeptide, of the present invention or the antibody or binding fragment or portion thereof raised against the adhesin(s) can also be

administered by injectable dosage forms of these materials in solution or suspension in a physiologically acceptable diluent with a pharmaceutical carrier. Such carriers include sterile liquids such as water and oils, with or without the addition of a surfactant and other pharmaceutically acceptable adjuvants. Illustrative oils are those of petroleum, animal, vegetable, or synthetic origin, for example, peanut oil, soybean oil, or mineral oil. In general, water, saline, aqueous dextrose and related sugar solution, and glycols such as, propylene glycol or polyethylene glycol, are preferred liquid carriers, particularly for injectable solutions.

In yet another aspect of the present invention, the adhesin(s) i.e., protein or polypeptide, of the present invention can be used as antigens in diagnostic assays for the detection of *M. hyopneumoniae* antibodies in swine. Alternatively, the detection of *M. hyopneumoniae* can be achieved with a diagnostic assay employing antibodies or binding fragments or portions thereof raised by such antigens. Such techniques permit detection of *M. hyopneumoniae* in a sample of tissue or body fluids of pigs such as blood, sputum, urine, nasal secretions, bronchial secretions or biopsied materials.

In one embodiment, the assay system comprises a sandwich or competitive format. Examples of suitable assays include an enzyme-linked immunosorbent assay, a radioimmunoassay, a gel diffusion precipitin reaction assay, an immunodiffusion assay, an agglutination assay, a fluorescent immunoassay, a protein A immunoassay, or an immunoelectrophoresis assay.

The following examples are offered by way of illustration and, not limitation, of the present invention.

EXAMPLE I

Materials and Methods

1. Chemicals and Reagents:

Antibodies to *M. hyopneumoniae* were produced by immunizing rabbits with immunogens prepared in rabbit muscle infusion medium supplemented with 20% rabbit serum according to the method of Ro et al., Comparison of *Mycoplasma hyopneumoniae* strains by serologic methods, 1983, *Am. J. Vet. Res.*, vol. 44, pp. 2087-2094, which disclosure is hereby incorporated by reference. Peroxidase-conjugated goat anti-rabbit IgG

was purchased from Cappel (Durham, NC). Peroxidase substrate (ABTS) was from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD). Fluorescein isothiocyanate (FITC)-conjugated antibodies against *M. hyopneumoniae* were supplied by B. Erickson, Iowa State University, Ames, Iowa. Various sugars and glycoconjugates were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated.

2. Mycoplasma Strains:

M. hyopneumoniae strains LI27, FA1, 2A3, and J, were compared for their adherence capability in the microtiter plate adherence assay. Strain 232 2A3 and 232 FA1 were cloned from strain 232 by filtration of a broth culture of strain 232 as described by Tully et al., Cloning and Filtration Techniques for Mycoplasmas. In: Razin et al., eds., *Methods in Mycoplasmology*, vol. 1, New York Academic Press Inc, 1983, which disclosure is hereby incorporated by reference, in Friis mycoplasmal medium as described by Friis, Some Recommendations Concerning Primary Isolation of *Mycoplasma suipneumoniae* and *Mycoplasma flocculare*, a survey, *Nord. Vet. Med.*, 1975, vol. 27, pp. 337-339, which disclosure is hereby incorporated by reference. Strain 232 of *M. hyopneumoniae* was originally reisolated from a pig inoculated with strain 11 as described by Zielinski et al., Adherence of *Mycoplasma hyopneumoniae* to Cell Monolayers, 1990, *Am. J. Vet. Res.*, vol. 51, pp. 339-343, which is hereby incorporated by reference. Strain J was originally obtained from the American Type Culture Collection No. 25934. Strain 144L SCI 3AF was isolated from a diseased pig in the laboratory and filter-cloned three times. It was known that strains LI27, 232 and 144L were pathogenic to pigs, whereas that strain J was nonpathogenic (Zielinski et al., 1990, *Am. J. Vet. Res.*, vol. 51, pp. 344-348, which disclosure is hereby incorporated by reference).

M. hyopneumoniae strain 232 LI27 was originally derived from strain 11. Passages 1 to 5 were cultured in Friis mycoplasmal medium and/or 20% acid-adjusted swine serum (Friis, cited elsewhere herein, which disclosure is hereby incorporated by reference) for 24 to 48 hours at 37°C. Mycoplasmas were harvested by centrifugation at 25,000 xg for 15 minutes. For adherence and adherence inhibition assays, the mycoplasmal pellets were resuspended to 1/10 of the original volume and further diluted in adherence buffer (RPMI 1640 medium containing 1% gelatin) to the appropriate Color changing units (CCU) before addition to microtiter plates. For production of mycoplasmal proteins required for

affinity chromatography, the pellets were further washed three times with phosphate-buffered saline (PBS, pH 7.2) and resuspended in PBS to 1/100 of the original volume. Color changing units (CCU) representing the number of mycoplasmas were determined by serial dilution in tubes containing Friis medium. For investigation of the effect of *in vitro* passage on adherence, the infected lung homogenate (LI27) was passaged in Friis medium. Cultures at passages 3, 10, 20, 30, 40, 50, 60 and 70 were collected, washed and adjusted to contain 12.5 and 25 μg/ml protein.

3. Growth and Preparation of Mycoplasmal Strains:

The four strains of *M. hyopneumoniae*, LI27, FA1, 2A3, and J, were inoculated into Friis medium at a 1:10 ratio and incubated for 48 hours at 37°C in a shaking water bath set at 50 rpm. Cultures were harvested by centrifugation at 23,000 xg for 15 minutes, washed several times with phosphate-buffered saline (PBS) solution. The total protein concentration of each culture was then determined. Thereafter, the cultures were diluted in RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO) to the same concentrations (12.5 and 25 μg/ml) for the four different strains and added to cilia-coated microtiter plates in accordance with adherence assay of the invention.

4. Monoclonal Antibodies and Fragmentation:

Monoclonal antibodies (Mab) to various antigens of *M. hyopneumoniae* was supplied by Barb Erickson (Veterinary Medical Research Institute, Iowa State University, Ames). For adherence inhibition, these Mabs in culture supernatants were concentrated about 12 times with ammonium sulfate. Since Mab F2G5 inhibited the adherence, this Mab was also produced in ascites and purified with affinity chromatography. Mab F2G5 was purified from ascites with the ImmunoPure® IgM purification kit (Pierce, Rockford, IL). This kit, containing buffers and an affinity support, has everything required to purify mouse IgM antibodies from ascites. Purification was conducted according to the instructions supplied with the kit. The purified F2G5 was resuspended in PBS and stored at -20°C for affinity chromatography, adherence inhibition, and fragmentation.

Fragmentation of F2G5 was performed with immobilized pepsin as described by Beale et al., 1982, *Comp. Biochem. Physiol.*, vol. 71B, pp. 475-482, which disclosure is hereby incorporated by reference. This digestion method generates predominantly $F(ab')_2$

fragments from mouse IgM; essentially no $(F_c)_{5\mu}$ can be produced (Beale et al., 1982, cited above). Briefly 2 mg of F2G5 was applied to a centricon™ 100 microconcentrator (Amicaon, Danvers, MA) and spin down for 40 minutes at 4°C. The retentate was washed twice with IgM $F(ab')_2$ digestion buffer (10 mM sodium acetate, 150 mM NaCl, 0.05% NaN_3, pH 4.5) and finally resuspended in 1 ml digestion buffer. A small column packed with 2 ml pepsin-agarose (Pierce, Rockford, IL.) was equilibrated with 10 ml of the digestion buffer and prewarmed at 37°C for 5 minutes. The column was added with 1 ml F2G5 and incubated at 37°C for 2 hours. The digest was eluted with 4 ml of the digestion buffer. The eluate was filtrated with a microsep™ 300 concentrator to remove nondigested IgM. The filtrate was further concentrated with a centricon™ 30 microconcentrator. The retentate mainly containing $F(ab')_2$ fragments was washed twice with PBS and resuspended in 0.5 ml PBS. $F(ab')_2$ fragments of Mab 80.1, an antibody against a 64K protein which did not inhibit the adherence of *M. hyopneumoniae* to porcine cilia, were prepared using a similar procedure as described above.

5. Preparation of Ciliated Cells:

Specific-Pathogen-Free (SPF) pigs free of *M. hyopneumoniae*, 7 to 12 weeks old, were obtained from a herd maintained at the Iowa State University Animal Resources Station. The herd was originally established from cesarean-born, isolation-reared swine and was a mixture of Yorkshire and Hampshire bloodlines. Swine tracheas were collected as described by Zielinski et al., Adherence of *Mycoplasma hyopneumoniae* to porcine ciliated respiratory tract cells, 1993, *Am. J. Vet. Res.*, vol. 54, pp. 1262-1269, which disclosure is hereby incorporated by reference. Briefly, the pigs were euthanatized, and the tracheas and lungs were exposed. The tracheas were clamped at the larynx and separated from the lungs by cutting at the bifurcation. Other connective tissues were trimmed from the outer surface of tracheas and then the tracheas were washed three times by immersing in RPMI 1640 medium before they were cut into small fragments. Ciliated cells were collected by scraping the inner surface of tracheas with a sterilized stainless steel laboratory spoon. Cilia were extracted from ciliated cells according to the method described in Tuomanen et al., Receptor analogs and monoclonal antibodies that inhibit adherence of *Bordetella pertussis* to human ciliated respiratory epithelial cells, 1988, *J. Exp. Med.*, vol. 168, pp. 267-277, which disclosure is hereby incorporated by reference.

Briefly, ciliated cells were suspended in 40 ml buffer containing 20 mM Tris, 10 mM EDTA, and 125 mM sucrose (TES, pH 7.2). Two washings with the TES buffer were conducted by centrifugation at 300 xg for 5 minutes. The cell pellet was resuspended in 6 ml AES (80 mM acetate, 10 mM EDTA, 125 mM sucrose, pH 6.8) buffer and incubated for 5 minutes at 25°C. Then, 0.2 M $CaCl_2$ was added to a final concentration of 10 mM. The mixture was vortexed for 10 minutes, diluted with 20 ml TES buffer and then centrifuged at 500 xg for 10 minutes. The sediment which contained mainly cell bodies was saved and the supernate containing cilia was harvested by centrifugation at 18,000 xg for 15 minutes at 4°C. The ciliary pellet was washed twice with phosphate-buffered saline (PBS) and stored at -70°C until use. Purity of cilia was ascertained by light microscopy. Protein concentration was determined using the BCA* protein assay reagent (Pierce, Rockford, IL) according to the instructions provided with the product.

6. Coating Plates:

Purified cilia was solubilized with sodium dodecyl sulfate (SDS, 1 mg/mg protein) at 37°C for 45 minutes. This preparation was further diluted with sodium carbonate buffer (0.1 M, pH 9.5) to a final concentration of 10 μg/ml protein. To each well of a flat bottom microtiter plate (Immulon®2, Dynatech Laboratories, Inc., Chantilly, VA) was added 100 μl of the solution. The plates were incubated overnight at room temperature and stored at -70°C without removal of the coating solution. Coated plates were stored at -70°C for at least one year without loss of adherence ability. Porcine albumin (fatty acid free, 10 μg/ml) and gelatin (10 μg/ml) were coated onto plates under conditions used for cilia as negative controls. After deciliation, cell bodies of ciliated cells were also solubilized and immobilized onto microtiter plates (10 μg/ml protein).

7. Adherence and Adherence Inhibition Assays:

M. hyopneumoniae whole cells or lysate (2×10^8 CCU/well for whole cells and 10 μg/ml for lysate) were added into microtiter plates coated with SDS-solubilized porcine tracheal cilia and incubated at 37°C for 90 minutes. The mycoplasmas or mycoplasmal proteins attached to the plates were detected by sequential addition of rabbit anti-*M. hyopneumoniae* hyperimmune antibodies and goat anti-rabbit Ig conjugated with peroxidase. For inhibition assays, various monoclonal antibodies of $F(ab')_2$ fragments

were preincubated with intact mycoplasmas at 37°C for 60 minutes. Two washings with PBS were conducted to remove free antibodies. The preincubated mycoplasmas were added into cilia-coated plates, and adherence was measured as described elsewhere herein. Percentage of inhibition was calculated as follows: % inhibition = (OD from mycoplasmas treated with dilution buffer - OD from mycoplasmas treated with Mabs)/OD from mycoplasmas treated with dilution buffer. Purified adhesins by Mab F2G5 affinity chromatography were evaluated for inhibition of the adherence of intact mycoplasmal cells. For this purpose, cilium-coated plates were first incubated with the purified adhesin at 37°C for 90 minutes. Dilution buffer (PBS containing 1% gelatin) was utilized as the noninhibitory control. After 4 washings with PBS, intact *M. hyopneumoniae* cells were added to the plates and incubated at 37°C for 90 minutes. The attached mycoplasmas were detected with a monospecific rabbit antiserum (R409) against 64K protein of *M. hyopneumoniae* (Young et al., 1987, *Am. J. Vet. Res.*, vol. 48, pp. 651-656, which disclosure is hereby incorporated by reference. It was determined in a preliminary experiment that antiserum R409 did not cross-react with the purified adhesins, but reacted with whole cells of the mycoplasma.

8. Statistics:

Analysis of variance with the Tukey contrast at a significance level of 0.05 was utilized for comparison among multiple treatments in an assay. The Student test was used if only two treatments were evaluated.

9. Comparison of Different Strains for Adherence Activities:

Various strains of *M. hyopneumoniae* were cultured in Friis mycoplasmal medium, harvested by centrifugation, and washed with PBS as previously described. The protein concentration of each strain was adjusted to 25 μg/ml for adherence assay. The protein patterns of different strains were analyzed by SDS-PAGE and by immunoblotting with Mab F2G5.

EXAMPLE II

Microtiter Plate Adherence Assay

After 4 washings with PBS (pH 7.4), the cilia-coated plates were blocked with 200 μl/well of AB for 2 hours at 37°C. Then, 100 μl of *M. hyopneumoniae* cells resuspended in AB were added to each well and the plates were incubated at 37°C for 90 minutes. Non-adherent mycoplasmas were removed by four washings with PBS. Subsequently, 100 μl of rabbit antibodies to *M. hyopneumoniae* and 100 μl of goat anti-rabbit peroxidase conjugates were sequentially added and incubated for 60 minutes at 37°C. Binding of mycoplasmas was visualized by addition of ABTS. Optical density (OD) values were measured with an automated microplate reader (Model EL310, Bio-Tek Instruments, Inc., Winooski, VT). Binding of mycoplasmas to cell body-coated plates was detected as described elsewhere herein.

M. hyopneumoniae bound specifically to cilia-coated wells but not to control wells coated with gelatin or porcine albumin (Figure 6). OD values obtained with the control wells were always less than 0.08. The degree of binding was influenced by both the number of mycoplasmas and the concentration of cilia utilized for coating plates. The optimum concentration of cilia for coating plates was 1 μg protein per well. About 2×10^8 mycoplasmas were required for half maximum binding at 37°C. Saturated binding was observed with more than 1×10^9 CCU mycoplasmas per well. Compared to 37°C, the binding activity of *M. hyopneumoniae* was about 3 times lower at 25°C and minimal at 4°C. The adherence was also time-dependent, with an optimum incubation time of 90 minutes (data not shown). Prolonged incubation resulted in increased background values. Well to well or plate to plate difference was negligible (<5%). Mycoplasmas resuspended in PBS or RPMI 1640 medium adhered equally well to cilia-coated plates (data not shown), although the latter was utilized throughout this procedure. Mycoplasmas solubilized by SDS or sonication also had the ability to adhere. In fact, solubilized mycoplasmas had higher adherence activity than intact mycoplasmas (date not shown). Heating of mycoplasmas at 56°C for 30 minutes resulted in an 80% reduction in adherence. With increased passage level, *M. hyopneumoniae* gradually decreased in adherence activity (Figure 2), with a significant decrease starting at passage 50 ($P<0.01$).

Mycoplasmas bound equally well to cell body-coated wells and to cilia-coated wells (data not shown).

EXAMPLE III

Adherence Inhibition

Various carbohydrates and glycoconjugates were evaluated for inhibition of adherence as shown in Table 1.

TABLE 1

Name	I^{50a}
Asialofetuin	*[b]
Asialomucin (bovine submaxillary glands)	*
Chondroitin sulfate A	80
Chondroitin sulfate B	20
Chondroitin sulfate C	-[c]
D-fucose	-
D-galactose	-
D-galactose-6-sulfate	-
D-glucose	-
D-glucose-6-sulfate	-
D-mannose	-
Dextran	-
DEAE dextran	-
Dextran sulfate MW 5,000	<1
Dextran sulfate MW 8,000	5
Dextran sulfate MW 500,000	<1
Diacetylchitobiose	-
Fetuin	*
Fucoidan	<1
Galactopyranosyl-galacto-pyranose	-
Heparin	<1 unit/ml
Hyaluronic acid	-
L-fucose	-
Lactose	-
Laminin	50
Lactoferrin	-
Melibiose	-
Mucin (bovine submaxillary glands)	*
Mucine (porcine intestine)	40
N-acetyl-galactosamine	-

N-acetyl-glucosamine	-
N-acetyl-neuramin-lactose	-
N-acetyl-mannosamine	-
N-acetyl-lactosamine	-
N-acetyl-glucosamine-3-sulfate	-
N-acetyl-glucosamine-6-sulfate	-
Sialic acid	-
$MgSO_4$	-
Methionine	-
Tetramethyl urea	-

([a]) Concentration (μg/ml, unless indicated) that resulted in 50% inhibition.
([b]) Partial inhibition was observed, but not more than 50% at the maximum concentration (2 mg/ml) tested.
([c]) No inhibition was detected at concentrations of up to 2 mg/ml.

The carbohydrates and glycoconjugates were diluted to appropriate concentrations in Ab, mixed with mycoplasmas and added to cilia-coated plates. After incubation and washings, rabbit anti-*M. hyopneumoniae*, and goat anti-rabbit conjugates were added as described previously. Two approaches were utilized to study competitive inhibition; 1) competitors were preincubated with mycoplasmas at 37°C for 1 hour, unbound competitors were removed by differential centrifugation (25,000 xg for 15 minutes), and incubated mycoplasmas were applied to cilia-coated plates; 2) cilia-coated plates were preincubated with competitors at 37°C for 1 hour, unbound competitors were removed by four washings, then mycoplasmas were added to the plates. Thereafter, procedures for both approaches were identical, including sequential addition of antibodies to mycoplasmas, secondary antibody conjugates and peroxidase substrates. Percentage of inhibition was calculated as follows: % inhibition = (OD value from AB - OD value from an inhibitor)/OD value from AB.I^{50} representing the concentration of a competitor that resulted in 50% inhibition was determined from the inhibition kinetics obtained with multiple concentrations of a competitor.

Since host cell surface receptors for mucosal pathogens are usually carbohydrate in nature, the binding of the mycoplasma in the presence of various sugars and glycoproteins was evaluated. In order to facilitate screening, competitors and mycoplasmas were added at the same time to cilia-coated plates. Among the carbohydrates and glycoconjugates evaluated were dextran sulfate, heparin, fucoidin, chondroitin sulfate, mucin and laminin

inhibited adherence of *M. hyopneumoniae* (Table 1). Three different molecular weights of dextran sulfate produced similar inhibition kinetics and inhibited adherence as much as 90%, but dextran was not an efficient competitor. Other sulfated compounds including D-galactose-6-sulfate, D-glucose-6-sulfate, N-acetyl-glucosamine-3-sulfate and N-acetyl-glucosamine-6-sulfate had no effect on binding of *M. hyopneumoniae*. Magnesium sulfate, methionine and tetramethyl urea, which disrupts hydrophobic interactions, did not inhibit adherence. Mucin from porcine intestine produced 75% inhibition. At concentrations of 20, 200 and 1,000 μg/ml, mucin from bovine submaxillary glands produced substantially lower inhibition than mucin from porcine intestine (data not shown). Actually, inhibition by bovine mucin never exceeded 30% at concentrations of up to 2 mg/ml. Remarkable differences in inhibition were obtained among chondroitin sulfate A,B or C. Chondroitin sulfate B and chondroitin sulfate A had an I^{50} of 20 μg/ml and 80 μg/ml, respectively, while no inhibition occurred with chondroitin sulfate C at concentrations of up to 2 mg/ml. Sialic acid had no effect on adherence. Partial inhibition (<50%) was obtained with fetuin, a glycoprotein rich in sialic acids. Asialofetuin and asialomucin, which were derived from fetuin and mucin (bovine submaxillary glands), respectively, and chemically modified to remove surface sialic acids, had significantly higher inhibition than fetuin and mucin (Table 2).

TABLE 2

Concentration (μg/ml)	% inhibition[a] by:			
	Mucin	Asialomucin	Fetuin	Asialofetuin
10	2.9 ± 2.9	6.6 ± 4.8	ND	ND
50	ND	ND	3.9 ± 7.1	19.3 ± 4.6[b]
100	25.9 ± 3.3	30.9 ± 1.6[c]	ND	ND
500	ND	ND	27.2 ± 4.3	48.9 ± 3.4[b]

([a]) Mean % inhibition ± SD in three experiments.
([b]) P<0.01 versus fetuin as determined by student t test.
([c]) P<0.05 versus mucin as determined by student t test.

EXAMPLE IV

Single Ciliated Cell Adherence Assay

Substances determined to have inhibitory activity with the microtiter plate adherence assay of the invention were further evaluated using the Single Ciliated Cell Adherence Assay according to the procedure of Zielinski et al., Adherence of *Mycoplasma hyopneumoniae* to porcine ciliated respiratory tract cells, 1993, *Am. J. Vet. Res.*, vol. 54, pp. 1262-1269, which disclosure is hereby incorporated by reference. Briefly, single ciliated cells were brushed off the epithelial surface of a trachea from a SPF pig and resuspended in RPMI 1640 medium (Sigma Chemical Co., St.Louis, MO). Cells were washed, counted and resuspended in the medium to 10^5 cells/ml. One ml of ciliated cells was mixed with 25 μl of mycoplasmas (10^{10} CCU/ml) and 25 μl of inhibitor diluted to the appropriate concentration (Table 4). After incubation in a water bath for 90 minutes, free mycoplasmas were removed by centrifugation (500 xg for 10 minutes). Cell pellets with associated mycoplasmas were washed twice with PBS and pipetted to glass slides. Air-dried, methanol-fixed slides were stained with FITC-conjugated antibodies to *M. hyopneumoniae* and observed under UV light with a Nikon epifluorescence microscope. The fluorescent score (an estimation of the number of mycoplasmas bound to a ciliated cell) for each sample was calculated as reported by Zielinski et al., cited above, which disclosure is hereby incorporated by reference.

It was speculated that positive results obtained in the adherence assay with various carbohydrates and glycoconjugates could have resulted either by interference with adhesins of mycoplasmas or by interference with receptors on cilia. The effects of the six competitors were further evaluated by preincubating them with mycoplasmas or with cilia. When preincubated with mycoplasmas, fucoidin, heparin, chondroitin sulfate B and mucin significantly blocked binding of the mycoplasma; laminin (6%) and dextran sulfate (16%) resulted in limited inhibition, as shown in Table 3.

TABLE 3

Treatment (μg/ml)	% inhibition[a] produced by preincubation with	
	cilia	mycoplasmas
Laminin (200)	74.8 ± 0.6[b]	6.2 ± 5.8
Mucin (200)	-6.6 ± 6.6	78.3 ± 3.1
Dextran sulfate MW 500,000 (100)	0.5 ± 6.7	16.4 ± 20.2
Chondroitin sulfate B (200)	-1.3 ± 0.4	45.6 ± 15.5
Fucoidan (100)	27.0 ± 3.5	77.6 ± 6.8
Heparin (10 units/ml)	-2.2 ± 6.2	93.4 ± 11.5

([a]) % inhibition was calculated versus adherence buffer.
([b]) Mean % inhibition ± SD in triplicated assays.

When the six competitors were pre-incubated with cilia, laminin produced 75% inhibition, whereas fucoidan produced 27% which was much less than that from preincubation with mycoplasmas; the other 4 competitors had no inhibitory effect. The six competitors also significantly reduced attachment of *M. hyopneumoniae* to intact ciliated cells in the single ciliated cell adherence assay, as shown in Table 4.

TABLE 4[a]

Inhibitor (μg/ml)	Adherence (fluorescence score)[b]
RPMI 1640	21.6 ± 6.0
Dextran sulfate MW 500,000 (10)	1.8 ± 1.4[c]
Heparin (10 units/ml)	8.2 ± 4.7[c]
Laminin (200)	12.3 ± 5.3[c]
Fucoidan (100)	1.1 ± 0.8[c]
Mucin (200)	2.1 ± 1.7[c]
Chondroitin sulfate B (100)	9.4 ± 4.9[c]

([a]) Adherence was conducted with the SCCAA model in the presence of the inhibitors.
([b]) Means ± SD in duplicate experiments.
([c]) Significantly different from RPMI 1640 as determined by analysis of variance with the Tukey contrast ($P<0.05$).

EXAMPLE V

Treatment Of Cilia With Neuraminidase And Sodium Metaperiodate

Purified cilia were incubated with 10 mM or 100 mM sodium metaperiodate in 10 mM sodium acetate (pH 5.0) at 37°C for 30 minutes, then washed three times with PBS, solubilized with SDS, and coated onto microtiter plates according to the procedures previously described herein. Cilia treated with 10 mM sodium acetate only were utilized as the controls. Adherence of *M. hyopneumoniae* to treated cilia was evaluated according to the procedures described herein. For treatment with neuraminidase, purified cilia were incubated with neuraminidase (1 unit/ml in 10 mM sodium acetate, pH 5.0) at 37°C for 30 minutes. After three washings with PBS, the treated cilia were solubilized with SDS and coated onto microtiter plates. Adherence was evaluated according to the procedures described herein.

Pretreatment of cilia with neuraminidase resulted in a trend toward increased binding of *M. hyopneumoniae* (Figure 7), although the increase was not significant. In contrast to neuraminidase, pretreatment of cilia with sodium metaperiodate, which perturbs carbohydrate structure, significantly reduced attachment (Figure 7).

EXAMPLE VI

Effect of Passage Level on Adherence

Infected lung homogenate (LI27) was successively passaged in Friis medium until passage 70. Cultures at intervals of ten passages were collected, washed, and adjusted to the same concentrations (12.5 μg/ml and 25.0 μg/ml) of proteins for different passages. Adherence was conducted in the cilia-coated microtiter plate adherence assay of the invention as described elsewhere herein.

EXAMPLE VII

Selection of High-Adherent and Low-Adherent Clones

Single colonies of *M. hyopneumoniae* strain LI27 obtained on agar plates were picked and inoculated into tubes containing 2 ml Friis medium. After growth was exhibited, the clones were frozen at -70°C. For the microtiter plate adherence assay, individual clones were thawed and incubated in Friis medium. After 24 to 48 hours incubation, adherence activities of each individual clone were evaluated in the microtiter plate adherence assay. Stability of high-adherent clones and low-adherent clones were determined by subcloning and *in vitro* passage. Protein profiles were compared by SDS-PAGE and immunoblotting.

EXAMPLE VIII

Adherence Inhibition by Monoclonal Antibodies

A library of monoclonal antibodies (prepared by B. Erickson, Iowa State University, Ames, Iowa) with different specificity to *M. hyopneumoniae* proteins was utilized to block adherence in the microtiter plate adherence assay. Monoclonal antibodies were separately incubated with mycoplasmas for 60 minutes at 37°C. Free antibodies were removed by centrifugation. Mycoplasmas with bound monoclonal antibodies were resuspended and added to microtiter plates coated with cilia for adherence studies.

EXAMPLE IX

Affinity Chromatography

Low passages of strain LI27 of *M. hyopneumoniae* were utilized for preparation of mycoplasmal adhesin(s)(proteins). Strain LI27 was cultured in Friis medium for 48 hours at 37°C. Mycoplasmas were harvested by centrifugation and washed three times with PBS solution. Thereafter the mycoplasmas were resuspended in PBS and solubilized with CHAPS, a nondenaturing zwitterionic detergent. Particles or fragments were removed by ultracentrifugation at 100,000 xg for 60 minutes. The supernate containing mycoplasmal adhesin(s) (proteins) was added to a affinity chromatography column packed with heparin-

agarose beads. The void fraction was removed by extensive washing with PBS solution, and captured proteins were eluted off the column with 0.7M NaCl or a stepwise gradient of NaCl. Adherence activity of various fractions was analyzed using the adherence assay of the invention. Protein patterns were evaluated by SDS-PAGE and immunoblotting.

EXAMPLE X

SDS-Page and Immunoblotting

SDS-PAGE was performed according to the procedure of Laemmli, U.K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4, 1970, *Nature*, vol. 227,pp. 680-685, which disclosure is hereby incorporated by reference, with 10% separating gel and 4% stacking gel. Fractionated components were electrophoretically transferred to nitrocellulose membrane according to the procedure of Towbin et al., Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheet: Procedure and some applications, 1979, *Proc. Natl. Acad. Sci. USA*, vol. 76(9), pp. 4350-4354, which disclosure is hereby incorporated by reference. After reaction with convalescent serum, the blots were incubated with peroxidase-labeled anti-swine IgG, and then exposed to peroxidase substrate.

EXAMPLE XI

Assay for Screening for High Adherent Clones

A low passage of pathogenic *M. hyopneumoniae* will be diluted in Friis broth medium, and dilutions will be inoculated onto Friis agar plates. The agar plates will be incubated at 36°C in a candle jar for 7-10 days. Colonies will be picked and inoculated into individual tubes of Friis broth medium and incubated. Mycoplasma cells harvested from cultures of single colony isolates will be tested in the microtiter plate adherence assay. The subclones with high adherence activity will be selected and recloned.

EXAMPLE XII

Assay for Screening for Adhesin-Based Vaccine

Each batch of adhesin-based vaccine will be evaluated in the microtiter plate adherence assay to assure that each batch will have the equivalent level of adherent activity. The adhesin-vaccine preparation will be added to the cilia-coated plate in place of the *M. hyopneumoniae* cells to test the adherent activity.

EXAMPLE XIII

Surface Proteolysis of Mycoplasmas with Enzymes

Proteolytic digestion of intact mycoplasmas was performed as described by Franzoso et al., 1993, *Infect. Immun.*, vol. 61, pp. 1523-1530, which disclosure is hereby incorporated by reference. *M. hyopneumoniae* strain LI27 grown in Friis mycoplasmal medium was harvested and washed three times with PBS by centrifugation. The mycoplasmas were resuspended to 0.5 mg/ml (protein concentration) in PBS. One ml of mycoplasmas was incubated with graded doses (final concentrations: 0, 0.01, 0.04, 0.1, 0.25, 1, 5, 25, and 100 μg/ml) of trypsin (type XI, DPCC treated, Sigma Chemical Company, St. Louis, MO) at 37°C for 30 minutes. After digestion, trypsin inhibitor (type I-S, Sigma) was added to the mixture at a final concentration of 200 μg/ml to stop the reactions. The treated mycoplasmas were washed three times in PBS and resuspended in 0.2 ml PBS. Each treated sample was titrated for CCU to determine the viability of treated mycoplasmas, evaluated for adherence activity by the microtiter plate assay, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with F2G5. Mycoplasmas were also treated with various doses (Figure 10) carboxypeptidase Y under similar conditions. The treated mycoplasmas were analyzed by SDS-PAGE and immunoblotting.

EXAMPLE XIV

Immunoelectron Microscopy

Immunolabelling of mycoplasmas with Mabs was performed as described by Almeida et al., 1991, *Infect. Immu.*, vol. 59, pp. 3119-3125, which disclosure is hereby incorporated by reference. *M. hyopneumoniae* cells resuspended in PBS containing 1% gelatin were incubated with 1:10 diluted F2G5 or with other Mabs at 37°C for 1 hour. The mycoplasmas were washed 3 times with PBS and then reacted with 1:10 diluted goat anti-mouse IgM (μ chain specific) conjugated with gold (10 nm, Sigma) at 37°C for 1 hour. After three washings with PBS, the mycoplasmas were fixed with 3% glutaraldehyde-cacodylate buffer, dehydrated in acetone, and embedded in epoxy resin. There was no osmium tetroxide postfixation step after fixation with glutaraldehyde. Thin sections were stained with uranyl acetate and lead citrate and observed under a Hitachi H500 electron microscope.

EXAMPLE XV

SDS-PAGE and Immunoblotting

SDS-PAGE was performed according to the method of Laemmli, 1970, cited elsewhere herein, which disclosure is hereby incorporated by reference, with 10% separating gel and 4% stacking gel. Mycoplasmas or mycoplasmal proteins were mixed with equal volumes of treatment buffer (0.125 M tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol) and heated at 100°C for 3 minutes prior to electrophoresis. The separated proteins were visualized by staining the gels with Coomassie brilliant blue R-250. For immunoblotting, proteins on the gels were transferred to nitrocellulose membrane as described by Towbin et al., 1979, cited elsewhere herein, which disclosure is hereby incorporated by reference. The blots were blocked with 3% BSA, 0.1% Tween 20 in PBS at 25°C for 60 minutes with gentle shaking. Then, the blots were incubated with appropriately diluted Mab F2G5 at 25°C for 2 hours. After three washings the PBS containing 0.01% Tween 20, the blots were further reacted with 1:1000 diluted goat anti-mouse Ig conjugated with peroxidase at 25°C for 2 hours. Three washings were performed prior to color development with 4-CN-α-naphthol.

EXAMPLE XVI

Antibody Affinity Chromatography

Mab F2G5 was coupled onto Affi-Gel®10 gel (Bio-Rad Laboratories, Melville, New York) according to the instructions supplied with the activated support. That is, 6 mg of Mab F2G5 resuspended in 4.5 ml of coupling buffer (0.1 M HEPES, pH 7.5) was mixed with 3 ml of Affi-Gel 10. This mixture was incubated at 4°C for 4 hours with gentle shaking. Then, 0.5 ml of 1M ethanolamine HCl (pH 8.0) was added and incubated at 4°C for 1 hour to stop the coupling. The gel was alternatively washed with 0.1 M Tris-HCl (Ph 7.5) and the coupling buffer. After packed into a small column, the gel was washed sequentially with 20 volumes of 10 mM Tris-HCl and 20 volumes of PBS containing 40 mM CHAPS (3-[(3-cholamindopropyl)-dimethyllammonio]-1-propane-sulfonate). *M. hyopneumoniae* strain LI27 was cultured, harvested, and washed as described above. The mycoplasmas resuspended in PBS containing 0.2 mM PMSF were solubilized with 40 mM CHAPS at 4°C for 1 hour. The lysate was ultracentrifuged at 100,000 *g* for 30 minutes. The supernate containing solubilized mycoplasmal proteins was harvested. Preliminary experiments showed that adhesins of *M. hyopneumoniae* were efficiently solubilized by CHAPS and that CHAPS did not interfere with the interaction between adhesins and Mab F2G5. Three ml of supernatant containing 12 mg of mycoplasmal proteins were applied to the column and reacted at 4°C overnight. Unbound proteins were washed out of the column with 50 ml of PBS containing 40 mM CHAPS. The captured proteins by the Mab were eluted with 0.1 M glycine-HCl (pH 2.5). The eluate was immediately neutralized to Ph 7.5 with 1 M Tris and concentrated to 1 ml with a centricon™ 10. The purity of the proteins was determined by SDS-PAGE and immunoblotting; the adherence activity of the eluted proteins was evaluated in the microtiter plate adherence assay; and the eluted proteins were also utilized to block the adherence of intact *M. hyopneumoniae* cells as described above.

EXAMPLE XVII

Digestion of Purified Adhesins with Trypsin

The controlled digestion of purified adhesins was performed as described by Rosengarten et al., 1990, *Science*, vol. 247, pp. 315-318, which disclosure is hereby incorporated by reference. The purified adhesins by F2G5-Affigel 10 were digested with graded doses of trypsin (0.04 to 1μg) at 37°C for 30 minutes. The digestion were immediately mixed with the treatment buffer and boiled at 100°C for 3 minutes. The samples from each digestion were pooled and subjected to SDS-PAGE and immunoblotting with Mab F2G5 as described above.

EXAMPLE XVIII

Protein Sequencing and Amino Acid Analysis

The N-terminal sequence of the 97K protein was determined as described by Matsudaira, P., 1987, J. Biol. Chem., vol. 262, pp. 10035-10038, which disclosure is hereby incorporated by reference. The purified adhesins by F2G5-affinity chromatography were further separated by SDS-PAGE. After electrophoresis was completed, the gel was washed three times with the transfer buffer (10 mM 3-(cyclohexylamino)-1-propanesulfonic acid, 10% methanol, pH 11). The proteins on the gel were electroblotted to polyvinylidene difluoride (PVDF) Immobilon-P membrane (Millipore) at 50 mA for 60 minutes. After rinse with distilled water, the membrane was stained with 0.1% Coomassie brilliant blue R-250 for 5 minutes. Destaining was conducted with 50% methanol, 10% acetate. Thereafter, the membrane was thoroughly washed with distilled water and air-dried. The membrane was submitted to the Protein Facility at Iowa State University where the 97K protein was excised from the membrane and subjected to automatic Edman degradation by using an Applied Biosystems 477A liquid-pulse sequencer. For amino acid analysis, the 97K protein was excised and analyzed with an Applied Biosystems 420 PTC amino acid analyzer.

EXAMPLE XIX

Adherence Inhibition by Mabs or Mab Fragments

Various Mabs were evaluated for inhibiting the adherence of *M. hyopneumoniae*, as shown in Table 5 as follows:

TABLE 5

Mab	Isotype	Specificity	Adherence inhibition
80.1	IgM	64	-
A1B2	IgM	100	-
A2H10	IgG1	100	-
F1B6	IgM	Multiple[b]	+
F2B11	IgG2a	86	-
F2G5	Igm	Multiple[b]	+
F3A6	IgG2b	116	-
F3B8	IgG1	105, 95, 65, 55	-
F3D5	IgG1	105, 95, 65, 55	-
F3D6	IgG1	105, 95, 65, 55	-
I1D8	IgG1	116	-
I2E12	IgG1	86	-
I2E12	IgG1	86	-
I3A6	IgG1	200, 145, 140, 116, 86	-
I4A10	IgG1	70	-
I4A4	IgG1	100, 90, 80, 60, 50	-
I4B1	IgG1	41, 43	-
I4B10	IgG1	41	-
I4B2	IgG1	41	-
I4E3	IgG1	116	-
R1E9	IgG3	40	-
R2E8	IgG1	67	-
R3G7	IgG1	116	-
R4B2	IgG1	40	-
R4B7	IgG2a	116, 105, 90, 80, 65, 60, 55	-
R5F4	IgG1	116	-
R6C10	IgG2a	116, 105, 90, 80, 65, 60, 55	-
R6E1	IgG1	116, 40	-
R6E4	IgG2a	116, 105, 80, 80, 65, 60, 55	-
R6G8	IgG1	46	-

\- no inhibition was detected with a 12 fold concentrated culture supernate.
\+ Produced does-dependent inhibition
[a] Sizes of antigens (in kilodaltrons) detected with strain LI27 on immunoblots; some Mabs detected antigens of varied sizes in different strains.
[b] Reacted with a set of proteins with distinct sizes, but predominantly recognized a 97 KDa protein

Two Mabs, F2G5 and F1B6, inhibited the adherence as much as 67%, as shown in Table 6 as follows:

TABLE 6

Dilution[a]	% inhibition[b] produced by Mabs		
	F3A6	F1B6	F2G5
1:2	-11.9 ± 3.1	67.0 ± 5.1	66.2 ± 4.5
1:10	2.5 ± 2.2	45.9 ± 3.4	58.7 ± 5.0
1:50	-11.1 ± 6.5	-10.8 ± 6.1	29.5 ± 3.7
1:250	-1.1 ± 2.4	-9.7 ± 8.0	13.0 ± 4.1

[a] Mabs were concentrated approximately 12 times from culture supernate and further diluted in the adherence buffer.

[b] Mean ± SD of three independent experiments.

As determined by immunoblotting, both Mabs had identical antigenic specificity, detected multiple bands, and reacted predominantly with a 97K protein (Figure 8). Several Mabs, such as R6C10, I3A6 and F3D5, which also detected multiple bands on immunoblots, did not inhibit the adherence (Table 5). The adherence-inhibiting activity was further evaluated with $F(ab')_2$ fragments of F2G5. As compared with the adherence buffer which was a negative control, the $F(ab')_2$ fragments of Mab F2G5 produced 60% inhibition on the adherence of *M. hyopneumoniae*, while no inhibition resulted with the F(ab')2 of Mab 80.1 (data not shown).

EXAMPLE XX

Binding of Solubilized Mycoplasmal Proteins to Cilia

Preliminary studies showed that CHAPS-solubilized mycoplasmal proteins adhered strongly to swine cilia. M. hyopneumoniae proteins solubilized with 40 mM CHAPS were ultracentrifuged to 100,000 xg to remove nonsolubilized debris. The supernate was diluted to 10 μg proteins/ml in PBS containing 1% gelatin and directly added to cilium-coated

plates for adherence assay. Detection of attached mycoplasmal proteins with various monoclonal antibodies demonstrated that the F2G5-reacting mycoplasmal proteins were capable of adherence, while other mycoplasmal proteins such as 41K, 64K, 100K and 116K did not attach to cilia, as shown in Table 7 as follows:

TABLE 7

Mab	Specificity[a]	OD value[b]
F3A6	116	0.039±0.003
F2G5	Multiple[c]	0.853±0.041
R6C10	105, 90, 80, 65, 60	0.053±0.006
80.1	64	0.038±0.009
I4B2	41	0.010±0.000
A2H10	100	0.026±0.001

[a] Sizes of antigens (in kilodaltons) detected in strain LI27
[b] Mean ± SD of triplicate experiments
[c] Reacted with multiple bands, but predominantly recognized a 97K protein

EXAMPLE XXI

Surface Proteolysis of Mycoplasmas

Treatment of *M. hyopneumoniae* with various doses (0.01 to 100 μg/ml) of trypsin did not significantly affect the viability of mycoplasmas as determined by CCU titration (data not shown). The 97K protein was extremely sensitive to trypsin. At low concentrations (0.01 to 1 μg/ml) of trypsin, the only visible change of mycoplasmal protein patterns on SDS-PAGE is the removal of 97K, 105K and 46K proteins (Figure 9). Immunoblotting of trypsin-treated mycoplasmas with F2G5 confirmed to the digestion of the 97K protein (Figure 9). Loss of the 97K protein was accompanied with the increased yield of 78K and 72K proteins (Figure 9). In fact, the 78K protein region contained a group of proteins with little difference in sizes ranging from 78K to 82K. 72K and 78K proteins were relatively insensitive to trypsin, but they also gradually digested at 25 to 100 μg/ml trypsin (data not shown). The adherence activity of *M. hyopneumoniae* was very sensitive to trypsin, as shown in Table 8 as follows:

TABLE 8

Concentration of trypsin (μg/ml)	Adherence (OD value)[a]
0.00	0.67±0.01
0.01	0.48±0.02[b]
0.04	0.47±0.02[b]
0.10	0.43±0.01[b]
0.25	0.32±0.01[b]
0.50	0.43±0.03[b]
1.00	0.71±0.01
5.00	1.25±0.03[b]
25.0	0.80±0.02[b]
100	0.60±0.02

[a] Mean ± SD in triplicate experiments.
[b] Significantly different from the non-treated control as determined by an analysis of variance with the Turkey contrast ($P<0.05$).

Mycoplasmas treated with 0.001 to 0.2 μg/ml trypsin had a decreased adherence capability, whereas increased adherence activity was observed with mycoplasmas treated with 5 and 25 μg/ml trypsin (Table 8). However, solubilized trypsin-treated mycoplasmas showed a linear decrease in the adherence to cilia as compared with the nontreated control (data now shown). The changes in protein patterns and adherence activities of *M. hyopneumoniae* treated with trypsin were highly reproducible under the conditions of this study. In contrast to trypsin, carboxypeptidase Y did not digest the 97K protein from the surface of *M. hyopneumoniae* at the maximum concentration of 125 μg/ml evaluated in this study (Figure 10).

EXAMPLE XXII

Strain Variation in Adherence

Five strains *M. hyopneumoniae* exhibited different adherence activities when evaluated using the MPAA (Figure 1). The adherence activity of strain J was substantially lower than those of the other strains, whereas strain 144L had the highest adherence activity among the 5 strains. One of the major differences revealed by SDS-PAGE among

the five strains was in the regions detected by Mab F2G5 (Figure 11). Several bands including 97K and 46K proteins were missing in strains J and 144L. The 116K protein was also absent in strain J. Like the 97k protein, the P46K protein was also sensitive to trypsin as shown in Figure 9. Immunoblotting with Mab F2G5 revealed that the 97K protein was missed in strain 144L and strain J, with the concomitant appearance of a 95K protein in J and a doublet (93K and 92K) in strain 144L that reacted with Mab F2G5 (Figure 11). Also, the 72K protein was hardly detectible in strain J and disappeared in 144L, but both J and 144L had an extra-band (approximately 69K) that reacted with the Mab (Figure 11).

EXAMPLE XXIII

Immunoelectron Microscopy

M. hyopneumoniae cells were reacted with F2G5 and goat anti-mouse IgM conjugated with gold to determine the distribution of the adhesins. Mab F2G5 stained the fuzzy surface structures of *M. hyopneumoniae* (Figure 12). The gold-labeling distributed randomly on the surface on mycoplasmas; it was not polarized at a specific region as demonstrated with the P1 adhesin of *M. pneumoniae*. Mycoplasmas reacted with the cell culture medium and conjugate were utilized as a negative control and did not display any labeling with the gold particles (Figure 12).

EXAMPLE XXIV

Affinity Chromatography

The eluate from the affinity column packed with affi-gel® 10-immobilized F2G5 was analyzed by SDS-PAGE and immunoblotting. The predominant protein captured by immobilized F2G5 from strain LI27 was the 97K protein (Figure 13). On immunoblots, the proteins in the eluate reacted specifically with Mab F2G5 (Figure 13). A ladder of proteins with distinct sizes was detected by F2G5 in close proximate to the 72K protein. These size variants had an approximate spacing of 2K. Mab F2G5 did not detect the 78K protein, but it did detect a 81K protein in the purified materials (Figure 13). The purified proteins adhered strongly to cilium-coated wells, but not to the control wells coated with

gelatin or albumin (Figure 14). The purified proteins also produced a dose-dependent inhibition of the adherence of intact *M. hyopneumoniae* cells to cilia, as shown in Table 9 as follows.

TABLE 9

Mycoplasma (CCU)	Adherence[a] in the presence of purified 97K			
	0.0 μg/ml	8 μg/ml	30 μg/ml	60 μg/ml
5×10^8	0.55±0.03	0.47±0.03	0.37±0.03[b]	0.24±0.02[b]
2×10^8	0.40±0.01	0.31±0.02[b]	0.20±0.02[b]	0.11±0.01[b]
9×10^7	0.21±0.01	0.15±0.01[b]	0.12±0.01[b]	0.07±0.01[b]

[a] Adherence of intact mycoplasma cells to swine cilia was performed in the microtiter plate adherence assay of the invention. Attached mycoplasmas were detected with a monospecific antiserum, that did not cross-react with the 97K protein, and a secondary antibody conjugated with peroxidase. Data was expressed as mean OD ± SD of duplicate experiments.

[b] Significantly different from the noninhibitory controls as determined by an analysis of variance ($P<0.05$).

EXAMPLE XXV

Size Variation

Since Mab F2G5 reacted with multiple proteins of *M. hyopneumoniae* from a single strain, the relationship among these proteins needed to be determined. One possibility was that these proteins were size variants. Size variation of a epitope-bearing proteins in mycoplasmas has been illustrated by increasing the population of mycoplasmas loaded for SDS-PAGE or by treatment of mycoplasmas with proteolytic enzymes (Rosengarten et al., 1990, cited elsewhere herein. Here, ladders of bands were demonstrated with Mab F2G5 when a five to ten fold greater load of LI27 was used for SDS-PAGE (Figure 15). It seemed that there were different sets of size variants (brackets in Figure 15) in a single strain; each one had a predominant protein. This reactivity of Mab F2G5 to multiple proteins was unchanged when SDS-PAGE was run under nonreducing conditions. Since size-variable antigens usually contain repetitive amino acid sequences and display periodic

structures when treated with trypsin, the purified 97K protein were also digested with trypsin. This treatment yielded a ladder of peptides that reacted with F2G5 (Figure 16). The minimum resolvable fragment of trypsin-digested adhesins was approximately 20.5K. The size difference between the peptides was the multiple of 0.5 KDa.

EXAMPLE XXVI

N-terminal Amino Acid Sequences of 97K and 145K

The N-terminal sequences of the 97K and 145K proteins were determined as described by Matsudaira, P., 1987, *J. Biol. Chem.*, vol. 262, pp. 10035-10038, which disclosure is hereby incorporated by reference. The 97K protein was purified by Mab F2G5-affinity chromatography. The 145K protein was purified by heparin-agarose column. Both proteins (97K and 145K) were further separated by SDS-PAGE. After electrophoresis was completed, the gel was washed three times with transfer buffer (10 mM 3-{cyclohexylamino}-1-propanesulfonic acid, 10% methanol, pH 11). The proteins on the gel were electroblotted to polyvinylidene difluoride (PVDF) Immobilon-P membrane (Millipore) at 50 mA for 60 minutes. After rinsing with distilled water, the membrane was stained with 0.1% Coomassie brilliant blue R-250 for 5 minutes. Destaining was conducted with a solution of 50% methanol and 10% acetate. Finally, the PVDF membrane was thoroughly washed with distilled water and air-dried. The membrane was submitted to the Protein Facility, Iowa State University, where the 97K and 145K proteins were excised form the membrane and subjected to automatic Edman degradation using an Applied Biosystems 477A liquid-pulse sequencer. The N-terminal amino acid sequence of the 97K protein, beginning with alanine, is expressed as follows: Alanine-Aspartic Acid-X-Lysine-Threonine-Aspartic Acid-Serine-Aspartic Acid-Lysine-Aspartic Acid-Proline-Serline-Threonine-Leucine-Arginine-Alanine-Isoleucine-Aspartic Acid-Glutamic Acid-Glutamine (SEQ.ID.NO.1). The X in position 3 indicated an ambiguous residue which was not identified.

The N-terminal amino acid sequence of the 145K protein, beginning with methionine, is expressed as follows: Methionine-Asparagine-Threonine-Lysine-Valine-Serine-Leucine-Glutamine-Tyrosine-Alanine-Lysine-Isoleucine-Serine (SEQ.ID.NO.2).

With reference to the 97K protein, the X in positions 3 indicated the ambiguous sites. This sequence was compared by GCG with all known sequences in BenBank, EMBL and Swiss-Prot. No homology was detected between this sequence and the sequences of known bacterial or mycoplasmal adhesins. However, some homology (about 40%) was detected with neural cell adhesion molecule L1 precursor. The significance of the homology was unknown. Analysis with peptide structure predicted that the N-terminus of the 97K protein was hydrophilic, surface-exposed, and flexible (data not shown). The amino acid composition of the 97K protein lacks cysteine, and the total composition is as shown in Table 10 as follows:

TABLE 10

Amino acid	Composition (Mol %)
Asp	17.46
Glu	16.28
Ser	6.18
Gly	5.71
His	0.77
Arg	1.85
Thr	4.54
Ala	8.70
Pro	4.10
Tyr	2.94
Val	3.25
Met	0.31
Ile	4.02
Leu	8.71
Phe	4.29
Lys	10.92

RESULTS

Among the four strains evaluated, strain J had the lowest adherence activity, while strains LI27 and 2A3 were similar in adherence, which was significantly higher than adherence for strains FA1 and J, as shown in Figure 1. With reference to Figure 2, it is

shown that with the increase of passage levels *in vitro*, the adherence activity of *M. hyopneumoniae* strain LI27 gradually decreased (Stars indicate the passage at which significant decrease in adherence was observed). At passage 50, significant reduction in adherence was observed. Variation in adherence activity was observed among the 81 clones of LI27. Clones 91 and 60 were high-adherent and low-adherent clones, respectively. Ten subclones of clone 91 were all strong in adherence activity, while all 10 subclones of clone 60 had very low adherence capability. Growth in Friis medium up to 30 passages did not change the adherence activity and protein profiles of clone 91. Immunoblotting revealed that clone 60 did not have the 145K protein which was present in clone 91.

With reference to Figure 3, total mycoplasmal proteins were separated by the affinity column. The first large peak detected with a UV monitor at 280 nm was void proteins. The small peak eluted with 0.7M NaCl contained proteins captured by heparin. Based on the OD values at 280 nm, most of the mycoplasmal proteins were in the void fraction. However, the small peak, containing eluted proteins, had higher adherence activity than the void proteins.

Adherence activity was further compared by standardization of each fraction. With reference to Figure 4, when assayed at 5 μg/ml proteins, the eluted proteins had seven times higher adherence activity than the void proteins. Analysis of different fractions by SDS-PAGE and immunoblotting revealed that protein patterns varied in different fractions. Several proteins (i.e., 200K, 185K, 145K, 116K, 100K, 97K, 57K and 43K) were present only in the eluted fraction while others were in the void fraction. As shown in Figure 5, three proteins (78K, 46K and 40K) were observed in both fractions (Lane 1, whole mycoplasmal proteins; Lane 2, void proteins; and Lane 3, proteins captured by heparin) while eight proteins were present only in the eluted fraction.

Stepwise elution with NaCl further separated the captured proteins into different peaks which had varied adherence activities and protein patterns. The first peak containing three proteins, 100K, 40K and a smear between 74K to 78K, did not have significant adherence activity. The peak containing the 145K protein had higher adherence activity than the other peaks. Elution with a linear gradient of NaCl gave results similar to those obtained with the stepwise elution.

Thirty-one monoclonal antibodies were evaluated for adherence inhibition in the adherence assay of the invention. Two of the monoclonal antibodies (i.e., F1B6 and F2G5) produced dose-dependent inhibition. Immunoblotting revealed that the two Mabs were identical and reacted with multiple proteins (145K, 116K, 105K, 100K, 97K, 78K, 76K and 36K) of *M. hyopneumoniae*.

It was previously reported in Zielinski et al., Adherence of *Mycoplasma hyopneumoniae* to Cell Monolayers, March 1990, *Am. J. Vet. Res.*, vol. 51, No. 3, pp. 339-343, which is hereby incorporated by reference, that strain J was unable to cause pneumonia in pigs. It was determined in accordance with the present invention (not shown) that strain LI27 was virulent for pigs. The results, as disclosed herein, have revealed that strain LI27 of *M. hyopneumoniae* had significantly higher adherence activity than strain J, as determined with the microtiter plate adherence assay (Figure 1). It was also demonstrated herein that adherence capability of the mycoplasma was decreased by *in vitro* passages (Figure 2), which parallels the decreased pathogenicity. These data provide indirect evidence to indicate that adherence is directly correlated with the virulence of *M. hyopneumoniae*. Eighty-one clones of strain LI27 demonstrated heterogeneity in adherence. *In vivo* infection may select high-adherent clones, while *in vitro* passages may result in overgrowing of low-adherent clones, thus leading to variation of pathogenicity between low and high passages of *M. hyopneumoniae*.

The present invention discloses eight novel, substantially pure mycoplasmal adhesins (proteins) i.e., 200K, 185K, 145K, 116K, 100K, 97K, 57K and 43K, which were specifically captured by heparin using the affinity chromatography technique described elsewhere herein. These eight adhesins had much stronger adherence activity than the void fraction adhesins. This indicates that adhesins are enriched by heparin-agarose beads. The protein patterns detected by the monoclonal antibodies F2G5 or F1B6 were not identical to those captured by heparin, but they shared 4 proteins, 145K, 116K, 100K and 97K. Although captured by heparin, the 100K protein was not an adhesin, because it did not have adherence activity after fraction with stepwise elution. Characterization of high-adherent and low-adherent clones revealed that the 145K and 97K proteins play an important role in adherence of *M. hyopneumoniae*. It was determined in a previous study by Young et al., Analysis of Virulence-Associated Antigens of *Mycoplasma hyopneumoniae*, 1992, IOM Letters, 2:321, which disclosure is hereby incorporated by

reference, that the 145K protein was associated with the virulence of the mycoplasma, and it gradually disappeared after *in vitro* passage. Loss of the 145K protein was also correlated to reduction of cytotoxicity of *M. hyopneumoniae* to ciliated cells. Therefore, it can be concluded that multiple adhesins are involved in adherence of *M. hyopneumoniae* and that the 145K protein is one of such adhesins.

Furthermore, this study demonstrated that the monoclonal antibody (Mab) F2G5 and its $F(ab')_2$ fragments, which recognized a predominant 97K protein in several strains of *M. hyopneumoniae* inhibited the adherence of *M. hyopneumoniae* to porcine cilia (Table 6); purified 97K protein and its size variants adhered to cilia and blocked the adherence of intact *M. hyopneumoniae* cells (Figure 14 and Table 9); treatment of mycoplasmas with trypsin removed the 97K protein and decreased adherence of *M. hyopneumoniae* (Figure 10 and Table 8); and immunoelectron microscopy demonstrated that Mab F2G5-reacting proteins were located on the surface of *M. hyopneumoniae* (Figure 12). Based on these results, it can be concluded that the 97K protein and its size variants are adhesins of *M. hyopneumoniae*. These findings strongly indicate the 97K protein is an important virulence factor of *M. hyopneumoniae.*

Sensitivity to surface digestion with trypsin confirmed the localization of the adhesins on the surface of mycoplasmas, as demonstrated with immunoelectron microscopy. F2G5 mainly stained the fuzzy structures on the mycoplasmas, suggesting that the fuzzy structures may be the adherent organelle of the mycoplasma. It was reported previously that the fuzz on *M. hyopneumoniae* bridge the interaction between mycoplasmas and cilia (Blanchard et al., 1992, *Vet. Microbiol.*, vol. 30, pp. 329-241; and Tajima et al., 1982, *Infect. Immun.*, vol.37, pp. 1162-1169), and that the fuzzy structures were only observed with a pathogenic strain but not with a strain that was nonadherent and nonpathogenic in pigs. These evidences, together with the information generated herein, strongly suggested that the fuzzy structures of *M. hyopneumoniae* play an important role in the adherence. The fuzz on the surface of streptococci, composed of M proteins and lipoteichoic acid, mediated the adherence of streptococci to eucaryotic cells (Ellen et al., 1974, *Infect. Immun.*, vol. 9, pp. 85-91; Ellen et al., 1972, *Infect. Immun.*, vol. 5, pp. 826-830; and Tylewska et al., 1988, *Curr. Microbiol.*, vol. 16, pp. 209-).

It was unlikely that the reactivity of Mab F2G5 to multiple proteins was caused by the impurity of the Mab. The hybridoma cell line that secretes F2G5 was subcloned three

times, and the secreted antibodies were detected as a homogenous IgM isotype. Also, Mab F1B10 which was produced by an independent hybridoma had identical antigenic specificity as F2G5. It was believed that the reactivity of F2G5 to multiple proteins was indeed due to the presence of the epitope in multiple antigens. In fact, the expression of an epitope on multiple proteins is very common to many mycoplasmas; including *M. hyorhinis* (Rosengarten et al., 1990, cited elsewhere herein; and Rosengarten et al., 1991, *J. Bacteriol.*, vol. 173, pp. 4782-4793), *M. pulmonis* (Watson et al., 1988, *Infect. Immun.*, vol. 56, pp. 1358-1363), *M. hominis* (Olsen et al., 1991, *Infect. Immun.*, vol. 59, pp. 1683-1689), *Ureaplasma urealyticum* (Watson et al., 1990, *Infect. Immun.*, vol. 58, pp. 3679-3688) and *M. fermentans* (Wise et al., 1993, *Infect. Immun.*, vol. 61, pp. 3327-3333) .

It was found that Mab F2G5 reacted with ladders of bands on immunoblots (Figure 15) and recognized a different predominant protein in different strains (Figure 11). Treatment of purified 97K protein yielded periodic peptides (Figure 16). These results suggested that *M. hyopneumoniae* proteins reacted with Mab F2G5 undergo both intraspecies and intrastrain size variation. Since the population of the mycoplasmas was rather heterogenous, isogeneic populations of *M. hyopneumoniae* derived from single clones will be required to thoroughly determine the size variation and its effect on adherence activity of *M. hyopneumoniae*.

The P1 adhesin of *M. pneumoniae* was sensitive to trypsin, while the 30K adhesin of *M. pneumoniae* and the MgPa adhesin of *M. genitalium* were resistant to trypsin (Razin et al., 1992, *J. Gen. Microbiol.*, vol. 138, pp. 407-422). Treatment of *M. pneumoniae* with trypsin removed two surface proteins (P1 and P2) and diminished the adherence of *M. pneumoniae* to human respiratory epithelium (Hu et al., 1977, *J. Exp. Med.*, vol. 145, pp. 1328-1343; and Hu et al., 1982, *Science*, vol. 216, pp. 313-315); regeneration of P1 led to the recovery of the adherence activity (Hu et al., 1977, cited above). Proteins 97K, 105K and 46K were three *M. hyopneumoniae* proteins that were very sensitive to surface proteolysis with trypsin (Figure 9), suggesting that they must be surface-exposed. Besides reactivity with Mab F2G5, the 105k protein also reacted with several other Mabs that did not inhibit the adherence of *M. hyopneumoniae* to cilia (Table 5). The 46K protein was absent in strain J which is a low-adherent and nonpathogenic strain. However, the 46K protein was also missing in strain 144L which is high-adherent and pathogenic in pigs

(Figure 11). A monoclonal antibody to the 46K protein did not inhibit the adherence of *M. hyopneumoniae* (Table 5). The function of the 46k and 105K proteins remain to be determined. The decrease of adherence produced by trypsin digestion was accountable to loss of the 97K protein. The increased adherence activity of mycoplasmas, which were treated with 5 and 25 μg/ml trypsin and had lost the 97k protein, can not presently be explained. The mycoplasmas treated with higher concentrations of trypsin became very sticky and hard to disperse, suggesting the membrane properties of mycoplasmas might be substantially changed. Therefore, the increased adherence might be a due to altered membrane properties rather than to an intrinsic increase in adherence ability. This was supported by the adherence results obtained with solubilized trypsin-treated mycoplasmas, which displayed a linear decrease in adherence to cilia with increased concentration of trypsin.

Degradation of the 97K protein resulted in the increased quantity of 78K protein (Figure 9). The region of the 78K protein was not a single protein but contained a set of proteins with little difference in size. Increased quantity of a 72K protein was also observed with the digestion of the 97K protein. Because mycoplasmas were washed after surface digestion, 72K and 78K proteins must be membrane-associated. Although higher concentrations of trypsin also resulted in gradual digestion of the 78K and 72K proteins, they were less sensitive to trypsin that the 97K protein. These results suggested that the 97K, 78K and 72K proteins reacting with Mab F2G5 were interrelated in structure. One possibility was that the 78K and 72K proteins were size variants of the 97K protein. The 97K protein might have repetitive amino acid sequences as shown with the M protein of *Streptococci* and the variable lipoproteins (Vlps) of *M. hyorhinis* (Hollingshead et al., 1986, *J. Biol. Chem.*, vol. 261, pp. 1677-1686; and Yogev et al., 1991, *EMBO J.*, vol. 10, pp. 4069-4079). This hypothesis was supported by the production of a ladder of peptides from the digestion of the purified 97K protein (Figure 16). It is known that the genes encoding for size-variable proteins, such as M proteins and Vlps, usually contain repetitive coding sequences, and that gain or loss of the repeats leads to expression of size-variable proteins (Hollingshead et al., 1986; and Yogev et al., 1991, both cited above). A similar mechanism may operate in the 97K protein. Molecular cloning and sequencing of the gene for the 97K protein will be required to confirm this hypothesis.

It is known that different strains of *M. hyopneumoniae* vary in pathogenicity for pigs (Zielinski et al., 1990, *Am. J. Vet. Res.*, vol. 51, pp. 344-348). Strains LI27 and 144L were pathogenic, while strain J was incapable of inducing pneumonia in pigs. Also, no mycoplasmas were isolated from lungs of pigs inoculated with Strain J, indicating that this strain had lost the ability to colonize the respiratory epithelium (Zielinski et al., 1990, cited above). Strains 232 2A3 and 232 FA1 were both pathogenic for pigs (Young et al., 1990, *Proc. 11th Congr. Intl. Pig Vet. Soc.*, p. 97; and Zielinski et al., 1990, cited above), although experimental infection with strain 232 FA1 had a prolonged incubation period and induced only a mild clinical disease (Young et al., 1990, cited above). It was found herein that strain J had substantially lower adherence activity than the other strains, whereas strain 144L had the highest adherence activity among the five strains evaluated in this study. As shown in Figure 11, the predominant proteins stained by Mab F2G5 in strains J and 144L were different in sizes from those in other strains. It appeared that the size variation of the 97K protein was related to the change in adherence capability. Also, the adherence ability of *M. hyopneumoniae* appeared to be correlated with the pathogenicity in pigs. Isogeneic strains, differing only in adherence, should be utilized to determine the correlation between the adherence and virulence of *M. hyopneumoniae*.

Carboxypeptidase cleaves a protein from the C-terminus (Hayashi et al., 1973, *J. Biol. Chem.*, vol. 248, pp. 2296-2302). Resistance of the 97K protein to digestion with carboxypeptidase Y suggested that the C-terminus of the protein was not accessible to the enzyme, and therefore was not at the external surface of *M. hyopneumoniae*. These results also implied that the 97K protein might be anchored in the membrane by the C-terminus, rather than the N-terminus as shown with the Vlp of *M. hyorhinis* (Yogev et al., 1991, cited elsewhere herein). It was unknown if the 97K protein was a lipoprotein. It appeared unlikely because the 97K protein was nonextractable with Triton X-114 (data not shown). Also, there was no cysteine in the 97K protein (Table 10), which usually is the site for acylation of a bacterial or mycoplasmal protein (Wise et al., 1992, Mycoplasmas: Molecular Biology and Pathogenesis, American Society for Microbiology, Washington, D.C.; and Sankaran, 1993, Bacterial Lipoproteins, p. 163-181, *In* M.J. Schlesinger (ed.), Lipid modification of proteins, CRC Press, Boca Raton). Although a large number of lipid-modified proteins were found in mollicutes (Wise et al., 1992, cited above), *M. hyopneumoniae* only had 4 lipoproteins with molecular weights ranging form 44 to

65K as demonstrated in Wise et al., 1993, cited elsewhere herein). Cysteine was also absent in two *M. pneumoniae* adhesins, P1 and P30 (Razin et al., 1992, cited elsewhere herein). These results suggested that the 97K protein differs in some features from the Vlps of *M. hyorhinis*, but similar to M protein of *streptococci* which is not acylated and anchored to cell wall by the C-terminus (Hollingshead et al., 1986, cited elsewhere herein).

Several mycoplasmal adhesins, such as P1 and P30 of *M. hyopneumoniae*, and P140 of *M. genitalium*, share considerable homology at both protein and DNA levels (Dallo et al., 1989, *Infect. Immun.*, vol. 57, pp. 1059-1065; Dallo et al., 1990, *Infect. Immun.*, vol. 58, pp. 4163-4165; and Razin et al., 1992, cited above). However, the N-terminal sequence of the 97K protein did not have any homology with the known mycoplasmal adhesins. This result indicated that the adhesins of *M. hyopneumoniae* may be quite distinct from other mycoplasmal adhesins. However, it is premature to make this conclusion because the N-terminal sequence obtained from this study represented only a small portion of the amino acid sequence of the 97K protein, and the internal sequence was not available for comparison yet. Analysis of the amino acid composition of the 97K protein indicated that there were no cysteine and tryptophan in the 97K protein (Table 10). However, this does not mean that the 97K protein does not contain tryptophan because this residue is easily destroyed at the acid hydrolysis step required for amino acid analysis (Walker et al., 1992, Characteristics and structure determination of proteins, p. 85-105. *In* Analysis of Amino Acids, Proteins and Nucleic Acids, Butterworth Heinemann, Oxford). Since asparagine and glutamine were deaminated to the corresponding carboxylic acids (Asp and Glu, respectively) as a consequence of the acid hydrolysis step (Walker et al., 1992, cited above), Asp and Glu (Table 10) represented a mixture of asparagine and aspartic acid and a mixture of glutamine and glutamic acid, respectively. It will not be possible to know the relative amount of each in the mixtures until the full amino acid sequence of the 97K protein has been determined.

The 97K protein and its size variants may not represent all of the adhesins of *M. hyopneumoniae* since Mab F2G5 did not completely inhibit the adherence, and removal of the 97K protein by trypsin did not abolish the adherence of *M. hyopneumoniae* to cilia. This is not surprising since bacteria and mycoplasmas usually have more than one adhesin. For example, *M. pneumoniae* have 3 adhesins, including P1, P30 (Razin et al., 1992, cited above); a recent study suggested that another surface protein (P90) was also an adhesin of

M. pneumoniae (Franzoso et al., 1993, *Infect. Immun.*, vol. 61, pp. 1523-1530); *Bordetella pertussis* posses 4 potential adhesins: filamentous hemagglutinin, pertussis toxin, periactin and fimbriae (Tajima et al., 1982, *Infect. Immun.*, vol. 37, pp. 1162-1169). These adhesins mediate the adherence of *B. pertussis* either to respiratory ciliated cells or to macrophage (Tajima et al., 1982, cited above). The interactions between multiple adhesins of an organism and the receptors on host cells provide for increased adherence specificity and strength. It is expected that other adhesins, other than those already recognized by Mab F2G5, of *M. hyopneumoniae* will be discovered.

The present invention also provides a microtiter plate adherence assay which is specific and reproducible for evaluation of adherence mechanisms of *M. hyopneumoniae*. When compared with other commercially available adherence assays, the assay of the present invention is more objective and sensitive. Although it does not appear feasible to screen large numbers of adherence competitors using the single ciliated cell adherence assay, many samples can be easily analyzed using the microtiter plate adherence assay of the invention, resulting in identification of receptor analogues. Furthermore, the nature of receptors may also be determined using the adherence assay of the invention.

The data disclosed herein has demonstrated that adherence of *M. hyopneumoniae* is temperature and dose dependent. Lysed mycoplasmas had higher adherence activity than intact mycoplasmas, indicating that viability of *M. hyopneumoniae* is not required for successful binding and that solubilization of mycoplasmal membranes may expose more binding sites. Similar findings were reported with *Mycoplasma gallisepticum* as described by Kahane et al., Attachment of mycoplasmas to host cell membranes, 1982, *Rev. Infect. Diseases*, vol. 4(suppl.), pp. S185-S192, which disclosure is hereby incorporated by reference. Unlike *M. pneumoniae*, which did not adhere well in nutrient-deficient medium (Krivan et al., Adhesion of *Mycoplasma hyopneumoniae* to sulfated glycolipids and inhibition by dextran sulfate, *J. Biol. Chem.*, vol. 264, pp. 9283-9288, which disclosure is hereby incorporated by reference, *M. hyopneumoniae* did not require glucose for binding because mycoplasmas resuspended in RPMI and PBS bound equally well. Heating (56°C for 30 minutes) mycoplasmas before adherence remarkably decreased binding, indicating that adhesins of *M. hyopneumoniae* are sensitive to heat. A high passage culture of *M. hyopneumoniae* was reported to be unable to cause pneumonia in pigs (Zielinski et al., Effect of growth in cell cultures and strain on virulence of *Mycoplasma hyopneumoniae*

for swine, 1990, *Am. J. Vet. Res.*, vol. 50, pp. 344-348, which disclosure is hereby incorporated by reference).

Attenuation of virulence through successive *in vitro* passages was also obtained with other mycoplasmas (Taylor-Robinson et al., Mycoplasmal adherence with particular reference to the pathogenicity of *Mycoplasma pulmonis*, 1981, *Isr. J. Med. Sci.*, vol. 17, pp. 599-603; and Collier et al., The changing pathogenicity of *Mycoplasma pneumoniae* with passage *in vitro* correlates of virulence, 1985, *Diagn. Microbiol. Infect. Dis.*, vol. 3, pp. 321-328, which disclosures are hereby incorporated by reference). According to the present invention, the adherence capability of *M. hyopneumoniae* to SDS-solubilized cilia was gradually decreased by passage in Friis medium (Figure 2). This appears to parallel the decrease in pathogenicity of the mycoplasma as described by Zielinski et al., Effect of growth in cell cultures and strain on virulence of *Mycoplasma hyopneumoniae* for swine, 1990, *Am. J. Vet. Res.*, vol. 50, pp. 344-348, which disclosure is hereby incorporated by reference.

Furthermore, six adherence inhibitors were identified using the adherence assay of the invention (Table 1). Four of them, fucoidan, heparin, chondroitin sulfate and mucin, bound to the ligant on the surface of mycoplasmas and therefore were receptor analogues. In addition, they are all sulfated glycoconjugates, which indicates that sulfonation of carbohydrate chains is important for receptor activity. However, sulfate alone was not sufficient for mycoplasmal binding since other sulfated carbohydrates including D-glucose-6-sulfate, D-galactose-6-sulfate, N-acetyl-glucosamine-3-sulfate, N-acetyl-glucosamine-6-sulfate and SO_4^{-2} had no effect on attachment.

The six adherence inhibitors identified were all polyanionic molecules. The only structural difference between chondroitin sulfate A and C is the position of the sulfate (Sigma Chemical CO., 1990, Sigma manual, Sigma Chemical CO., St. Louis, MO, which disclosure is hereby incorporated by reference). Chondroitin sulfate A inhibited adherence of the mycoplasma, whereas chondroitin sulfate C showed no adherence inhibition. This suggests that the inhibition is probably not due to ionic interaction since chondroitin sulfate A and chondroitin sulfate C have similar charge properties. Chondroitin sulfate B, which contains iduronic acid instead of glucuronic acid found in chondroitin sulfate A (Sigma Chemical CO., 1990, Sigma manual, Sigma Chemical CO., St. Louis, MO, which disclosure is hereby incorporated by reference), caused significantly higher inhibition that

chondroitin sulfate A. These results suggest that the binding affinity of *M. hyopneumoniae* to cilia is greatly affected by the nature of carbohydrates and positions of sulfate on carbohydrate chains.

It has been shown that the animal cell surface is covered by various forms of carbohydrates (Karlson, K.A., Animal glycolipids as attachment sites for microbes, 1986, *Chemistry and Physics of Lipids*, vol. 42, pp. 153-172, which disclosure is hereby incorporated by reference). Many viruses (Dimmock, N.J., Initial stages in infection with animal viruses, 1982, *J. Gen. Virol.*, vol. 59, pp. 1-22, which disclosure is hereby incorporated by reference), bacteria (Sharon et al., Bacterial adherence to cell surface sugars, 1981, *In* Ciba Foundation symposium 80, Adhesion and microorganism pathogenicity, p.119-141, Pitman Medical, London, which disclosure is hereby incorporated by reference) and bacterial toxins (Eidels et al., Membrane receptors for bacterial toxins, 1983, *Microbiol. Rev.*, vol. 47, pp. 596-620, which disclosure is hereby incorporated by reference) utilize eucaryotic cell surface carbohydrates as attachment sites, which is an essential process for establishment of colonization and production of toxic effects. In the present inventions, the inhibition of adherence by several carbohydrates and the decreased binding caused by treatment of cilia with sodium metaperiodate strongly suggest that the receptors for *M. hyopneumoniae* are sulfated glycoconjugates. Both sulfated complex carbohydrates and sialylated glycoconjugates were demonstrated in rat (Spicer et al., Complex carbohydrates of rat tracheobronchial surface epithelium visualized ultrastructurally, 1980, *Am. J. Anat.*, vol. 158, pp. 93-109, which disclosure is hereby incorporated by reference), dog and human (Spicer et al., Histochemistry of mucosubstances in the canine and human respiratory tract, 1971, *Laboratory Investigation*, vol. 25, p. 483-490, which disclosure is hereby incorporated by reference) respiratory epithelium and in bronchial goblet cells of pigs (DeBey et al., Histochemical and morphologic changes of porcine airway epithelial cells in response to infection with *Mycoplasma hyopneumoniae*, 1992, *Am. J. Vet. Res.*, vol. 53, pp. 1705-1710, which disclosure is hereby incorporated by reference). These data indicate that sulfated carbohydrates are available *in vivo* for attachment of pathogens that colonize respiratory epithelium.

Moreover, many types of eucaryotic cells have membrane-associated heparin or heparin-like polysaccharides, which facilitate cell-cell interactions and cell-extracellular

matrix interactions (Comper, W.D., Heparin (and related polysaccharides), 1981, Gordon and Breach Science Publishers, New York, pp. 131-162; and Rollins et al., Glycosaminoglycans in the substrate adhesion sites of normal and virus-transformed murine cells, 1979, *Biochem.*, vol. 18, pp. 141-148, which disclosures are hereby incorporated by reference). But, it is not known if ciliated cells have surface exposed heparin or heparin-like polysaccharides. *M. hyopneumoniae* may utilize this natural substratum for attachment. Dextran sulfate produced more than 90% inhibition when mixed together with mycoplasmas and cilia, but it caused limited inhibition (16%, Table 3) when preincubated with mycoplasmas. This discrepancy might be caused by the low affinity of dextran sulfate for mycoplasmas. After preincubation, dextran sulfate could be removed from mycoplasmas by the washing step in the adherence assay of the invention. Laminin, a glycoprotein with a large molecular weight, did not interact with molecules on mycoplasmas. It has been reported that laminin binds specifically to sulfated glycolipids (Roberts et al., Laminin binds specifically to sulfated glycolipids, 1985, *Proc. Natl. Acad. Sci. USA*, vol. 82, pp. 1306-1310, which disclosure is hereby incorporated by reference). Also, sulfated glycolipids are receptors for several species of mycoplasmas (Krivan et al., Adhesion of Mycoplasma pneumoniae to sulfated glycolipids and inhibition by dextran sulfate, 1989, *J. Biol. Chem.*, vol. 264, pp. 9283-9288; and Lingwood et al., Common sulfoglycolipid receptor for mycoplasmas involved in animal and human infertility, 1990, *Biology of Reproduction*, vol 43, pp. 694-697, which disclosures are hereby incorporated by reference). Perhaps, laminin binds to glycolipid receptors in swine cilia and therefore inhibits adherence of *M. hyopneumoniae*. Sialic acid has been reported to be a receptor site for bacterial (Ishikawa et al., Evidence for sialyl glycoconjugates as receptors for *Bordetella bronchiseptica* on swine nasal mucosa, 1987, *Infect. Immun.*, vol. 55, pp. 1607-1609, which disclosure is hereby incorporated by reference), viruses (McClintock et al., Viral receptors:expression, regulation and relationship to infectivity, 1984, *In* A.L. Notkins and M.B.A. Oldstone (ed.), *Concepts in viral pathogenesis*, pp. 97-101, Springer-Verlag, New York, which disclosure is hereby incorporated by reference) and mycoplasmas (Razin et al., Mycoplasma adhesion, 1992, *J. Gen. Microbiol.*, vol. 138, pp. 407-422; and Gesner et al., Sialic acid binding sites:role in hemagglutination by *Mycoplasma gallisepticum*, 1966, *Science*, vol. 151, pp. 590-591; which disclosures are hereby incorporated by reference). Results of the present invention have demonstrated that

sialic acid is not involved in adherence of *M. hyopneumoniae*. In contrast, removal of sialic acids from cilia promoted adherence of *M. hyopneumoniae* (Figure 7). This is correlated with increased inhibition from asialomucin and asialofetuin (Table 2). Sialic acid is usually on the terminal position in oligosaccharide chains. Removal of it may either change the charge properties on cilia or uncover more receptor epitopes for the mycoplasma. It is not known whether *M. hyopneumoniae* produces neuraminidase during infection.

Secretion of large amounts of mucus during mycoplasmal pneumonia of swine seems to be a host defense mechanism to trap *M. hyopneumoniae*. However, this mycoplasma causes extensive damage to cilia (Blanchard et al., Electron microscopic observation of the respiratory tract of SPF piglets inoculated with *Mycoplasma hyopneumoniae*, 1992, *Vet. Microbiol.*, vol. 30, pp. 329-341; Mebus et al., Scanning electron microscopy of trachea and bronchi from gnotobiotic pigs inoculated with *Mycoplasma hyopneumoniae*, 1977, *Am. J. Vet. Res.*, vol. 38, pp. 1249-1254; and Zhang et al., Attenuated pathogenicity of the lapinized strain of *Mycoplasma hyopneumoniae* in pigs, 1990, *J. Chn. Electron Microscopy Soc.*, vol. 9(1), pp. 5-9, which disclosures are hereby incorporated by reference) by unknown means, which compromises the major clearance system in the swine respiratory tract. Thus, adherence to mucin may promote the infection of *M. hyopneumoniae* since secretions can not be efficiently excluded from the respiratory tract due to malfunction of the muco-transportation system. DeBey et al., Histochemical and morphologic changes of porcine airway epithelial cells in response to infection with *Mycoplasma hyopneumoniae*, 1992, *Am. J. Vet. Res.*, vol. 53, pp. 1705-1710, which disclosure is hereby incorporated by reference, quantitated bronchial goblet cell secretions of pigs by image analysis of sections stained with high iron diamine/Alcian blue. The results revealed that bronchial goblet cells of pigs infected with *M. hyopneumoniae* contained significantly less sialomucin and more sulfomucosubstances than goblet cells of control pigs. This finding suggests that goblet cells of pigs altered production of type of mucosubstances in response to infection with the mycoplasma.

It is apparent that adherence of *M. hyopneumoniae* to swine ciliated cells is a complicated process. In order to reach ciliated cells, the mycoplasma must overcome the sweeping force and penetrate the mucus gel which covers the surface of epithelium. Receptor-ligand interactions as well as ionic or hydrophobic interactions may contribute to

this process (Zielinski et al., Adherence of *Mycoplasma hyopneumoniae* to porcine ciliated respiratory tract cells, 1993, *Am. J. Vet. Res.*, vol. 54, pp. 1262-1269, which disclosure is hereby incorporated by reference). Tetramethyl urea, which disrupts hydrophobic interactions, inhibited adherence in the single ciliated cell adherence model (Zielinski et al., cited above, which disclosure is hereby incorporated by reference), but it did not inhibit adherence in the microtiter plate adherence assay of the present invention (Table 1). Therefore, the adherence is mainly adhesin-ligand mediated since 1) *M. hyopneumoniae* did not bind significantly to microtiter plate wells without coated cilia (Figure 6); 2) receptor analogues inhibited the adherence by as much as 90%; and 3) tetramethyl urea, which disrupts hydrophobic interactions, did not interfere with adherence (Table 1). Based on the results from the present invention, it is concluded that hydrophobic interactions play a minor role in the adherence, if at all.

M. hyopneumoniae also attached to the cell body of ciliated cells in the microtiter plate adherence assay of the invention, indicating presence of receptors in the plasma membrane of ciliated cells. This is not surprising since the ciliary membrane is the continuous plasma membrane of ciliated cells. This finding was not consistent with results obtained by the single ciliated cell adherence assay in which this mycoplasma predominantly bound to the ciliary tuft and only a few to cell bodies (Zielinski et al., cited above, which disclosure is hereby incorporated by reference). This contradiction may be explained by varied accessibility of receptors on different locations of ciliated cells. These findings suggest that host receptors that interact with adhesins of a pathogen are not the sole factor for tissue tropism.

The six inhibitors obtained in accordance with the microtiter plate adherence assay of the invention also blocked the attachment of *M. hyopneumoniae* to intact ciliated cells (Table 4), indicating adherence mechanisms involved in the microtiter plate adherence assay were comparable with those operating in the single ciliated cell adherence assay. In the single ciliated cell adherence assay, intact ciliated cells instead of solubilized cilia were utilized to react with mycoplasmas. This result confirmed the specificity of the receptor analogues which interrupt the intimate associations between molecules on the surfaces of mycoplasmas and ciliated cells. Thus, these receptor analogues provide a valuable alternative approach for purification and characterization of mycoplasmal adhesins. In addition, novel approaches to the prophylaxis of mycoplasmal pneumonia of

swine may be developed by utilizing the receptor analogues. It this context, it has been reported by Aronson et al., Prevention of colonization of the urinary tract of mice with *Escherichia coli* by blocking bacterial adherence with methyl alpha-D-mannopyranoside, 1979; *J. Infect. Dis.*, vol. 139, pp. 329-332, which disclosure is hereby incorporated by reference, that mannose, a receptor analogue for type I fimbriated *E. coli*, successfully decreased episodes of cystitis when instilled with the organism into the mouse bladder.

Although the invention has been described in detail for the purpose of illustration, it is understood that such detail is solely for that purpose, and variations can be made therein by those skilled in the art without departing from the spirit and scope of the invention which is defined by the following claims.

WHAT IS CLAIMED:

1. An adhesin isolated from the microbe *Mycoplasma hyopneumoniae*.

2. An adhesin according to claim 1, wherein said adhesin is capable of binding to a receptor analogue of *Mycoplasma hyopneumoniae*.

3. An adhesin according to claim 1, wherein said adhesin facilitates adherence of *Mycoplasma hyopneumoniae* to a host cell.

4. An adhesin according to claim 1, wherein said adhesin is selected from the group consisting of a protein, polypeptide, glycoprotein and lipoprotein.

5. An adhesin according to claim 4, wherein said adhesin is a protein.

6. An adhesin according to claim 5, wherein said protein is a polypeptide having a molecular weight, as determined by SDS-PAGE and immunoblotting, of from about 10 to about 300 kilodaltons.

7. An adhesin according to claim 6, wherein said protein is a polypeptide having a molecular weight, as determined by SDS-PAGE and immunoblotting, of about 145 kilodaltons.

8. The adhesin according to claim 7, wherein said 145 kilodalton polypeptide comprises the following N-terminal amino acid sequence: Methionine-Asparagine-Threonine-Lysine-Valine-Serine-Leucine-Glutamine-Tyrosine-Alanine-Lysine-Isoleucine-Serine (SEQ.ID.NO.2).

9. An adhesin according to claim 6, wherein said protein is a polypeptide having a molecular weight, as determined by SDS-PAGE and immunoblotting, of about 97 kilodaltons.

10. The adhesin according to claim 9, wherein said 97 kilodalton polypeptide comprises the following N-terminal amino acid sequence: Alanine-Aspartic Acid-X-Lysine-Threonine-Aspartic Acid-Serine-Aspartic Acid-Lysine-Aspartic Acid-Proline-Serline-Threonine-Leucine-Arginine-Alanine-Isoleucine-Aspartic Acid-Glutamic Acid-Glutamine (SEQ.ID.NO.1).

11. An adhesin according to claim 1, wherein said adhesin is substantially purified.

12. A method of vaccinating porcine against infection by *Mycoplasma hyopneumoniae* comprising:

administering to porcine an effective amount of the adhesin according to claim 1.

13. A method according to claim 12, wherein said administering is selected from the group consisting of oral, intradermal, intramuscular, intravenous, intranasal, subcutaneous, and by application to mucous membranes.

14. A vaccine for preventing infection and disease of porcine by *Mycoplasma hyopneumoniae* comprising:

an adhesin according to claim 1; and

a pharmaceutically-acceptable carrier or adjuvant.

15. A vaccine according to claim 14, wherein said adhesin is selected from the group consisting of a protein, polypeptide, glycoprotein and lipoprotein or portion thereof, and having a molecular weight, as determined by SDS-PAGE and immunoblotting, of from about 10 to about 300 kilodaltons.

16. A vaccine according to claim 14, wherein said adhesin is substantially purified.

17. A method of vaccinating porcine against infection by *Mycoplasma hyopneumoniae* comprising:

administering to porcine an effective amount of the vaccine according to claim 14.

18. A method according to claim 17, wherein said administering is selected from the group consisting of oral, intradermal, intramuscular, intravenous, intranasal, subcutaneous, and by application to mucous membranes.

19. An isolated antibody or binding fragment thereof raised against the adhesin according to claim 1.

20. An antibody according to claim 19, wherein said adhesin is selected from the group consisting of a protein, polypeptide, glycoprotein and lipoprotein, said adhesin having a molecular weight, as determined by SDS-PAGE and immunoblotting, of from about 10 to about 300 kilodaltons.

21. An antibody according to claim 19, wherein said antibody is a monoclonal antibody.

22. An antibody according to claim 19, wherein said monoclonal antibody is F2G5.

23. An antibody according to claim 19, wherein said monoclonal antibody is F1B6.

24. A method of passively immunizing porcine infected with *Mycoplasma hyopneumoniae* comprising:

administering an effective amount of said antibody or binding fragment thereof according to claim 19 to porcine infected with *Mycoplasma hyopneumoniae*.

25. A method according to claim 19, wherein said administering is selected from the group consisting of oral, intradermal, intramuscular, intravenous, intranasal, subcutaneous, and by application to mucous membranes.

26. A composition for passively immunizing porcine infected with *Mycoplasma hyopneumoniae* comprising:

an isolated antibody or binding fragment thereof according to claim 19; and

a pharmaceutically-acceptable carrier.

27. A method of passively immunizing porcine infected with *Mycoplasma hyopneumoniae* comprising:

administering an effective amount of the composition according to claim 26 to porcine at risk of being infected with *Mycoplasma hyopneumoniae*.

28. A method according to claim 27, wherein said administering is selected from the group consisting of oral, intradermal, intramuscular, intravenous, intranasal, subcutaneous, and by application to mucous membranes.

29. A method for detection of *Mycoplasma hyopneumoniae* antibody in a sample obtained from swine comprising:

providing an adhesin according to claim 1 as an antigen;

contacting said sample with said antigen; and

detecting any reaction which indicates that *Mycoplasma hyopneumoniae* antibody is present in the sample using an assay system.

30. A method according to claim 29, wherein said adhesin is selected from the group consisting of a protein, polypeptide, glycoprotein and lipoprotein, said adhesin having a molecular weight, as determined by SDS-PAGE and immunoblotting, of from about 10 to about 300 kilodaltons.

31. A method according to claim 29, wherein said assay system is selected from the group consisting of an enzyme-linked immunosorbent assay, a radioimmunoassay, a gel

diffusion precipitin reaction assay, an immunodiffusion assay, an agglutination assay, a fluorescent immunoassay, an immunoassay based on an immunoglobulin binding protein, and an immunoelectrophoresis assay.

32. A method for detection of *Mycoplasma hyopneumoniae* in a sample of tissue or fluids comprising:

providing an antibody or binding fragment thereof according to claim 19;

contacting the sample with said antibody or binding fragment thereof; and

detecting any reaction which indicates that *Mycoplasma hyopneumoniae* is present in the sample using an assay system.

33. A DNA molecule encoding an adhesin according to claim 1.

34. A method for detection of *Mycoplasma hyopneumoniae* in a sample of tissue or fluids comprising:

providing a nucleotide sequence of the DNA molecule according to claim 33 as a probe in a nucleic acid hybridization assay;

contacting the sample with the probe; and

detecting any reaction which indicates that *Mycoplasma hyopneumoniae* is present in the sample.

35. A method for detection of *Mycoplasma hyopneumoniae* in a sample of tissue or fluids comprising:

providing a nucleotide sequence of the DNA molecule according to claim 33 as a probe in a gene amplification detection procedure;

contacting the sample with the probe; and

detecting any reaction which indicates that *Mycoplasma hyopneumoniae* is present in the sample.

36. A method of isolating a *Mycoplasma hyopneumoniae* adhesin from a preparation of mycoplasmas comprising:

attaching a receptor analogue or antibody specific to a substrate;

placing the substrate in a column;

passing the mycoplasma preparation containing the adhesin through the column such that adhesins to which the receptor analogue or antibody is directed will bind to the receptor in the column; and

eluting the adhesins from the column by adding a solution that disrupts binding of the adhesins to the receptor or antibody.

37. The method according to claim 36, wherein said receptor analogue is selected from the group consisting of dextran sulfates, fucoidin, heparin, laminin, porcine mucin, chondroitin sulfate and monoclonal antibodies which recognize *Mycoplasma hyopneumoniae* adhesin.

38. The method according to claim 36, wherein said receptor analogue is heparin.

39. The method according to claim 36, wherein said column is an affinity chromatography column.

40. The method according to claim 36, wherein said substrate is a support selected from the group consisting of paper, metal foils, polystyrene, polyesters [e.g., poly(ethylene tere-phthalate)], polycarbonates, cellulose esters (e.g., cellulose acetate), glass beads and agarose.

41. An affinity chromatography assay for the isolation of *Mycoplasma hyopneumoniae* adhesins from a preparation of mycoplasmas comprising:

providing a column containing agarose-heparin beads packed therein;

passing the mycoplasma preparation containing the adhesins through the column; and

eluting the adhesins from the column with a solution which disrupts binding of the adhesins to the beads.

42. A method for determining the adhesive characteristics of *Mycoplasma hyopneumoniae* adhesins comprising:

isolating *Mycoplasma hyopneumoniae* adhesins according to claims 36 or 41; and

determining the adhesive characteristics of the isolated *Mycoplasma hyopneumoniae* adhesins.

43. A hybridoma cell line producing the antibody or binding fragment thereof according to claim 19.

44. An *in vitro* microtiter plate adherence assay for *Mycoplasma hyopneumoniae* cells, comprising:

immobilizing substantially purified cilia containing receptors for *Mycoplasma hyopneumoniae* to the wells of a microtiter plate;

reacting the bound cilia with a suspension of *Mycoplasma hyopneumoniae* cells; and

detecting the presence of *Mycoplasma hyopneumoniae* cells bound to the cilia.

45. The assay of claim 44, wherein said detecting step further comprises:

reacting the adhered *Mycoplasma hyopneumoniae* cells with an antibody to *Mycoplasma hyopneumoniae*;

reacting labelled anti-immunoglobulin antibody with the bound antibody; and

assaying for the presence of label, thereby determining adherent *Mycoplasma hyopneumoniae* cells.

46. The assay of claim 44, wherein said antibody is a polyclonal antibody.

47. The assay of claim 46, wherein said polyclonal antibody is rabbit antibody to *Mycoplasma hyopneumoniae*.

48. The assay of claim 44, wherein said anti-immunoglobulin antibody is goat anti-rabbit antibody.

49. The assay of claim 44, wherein said label is an enzyme.

50. The assay of claim 49, wherein said label is peroxidase.

51. The assay of claim 44, wherein said *Mycoplasma hyopneumoniae* cells are suspended in an adherence buffer.

52. The assay of claim 51, wherein said adherence buffer is buffer RPMI 1640 medium containing 1% gelatin.

53. The assay of claim 44, wherein said cilia are purified from ciliated trachea cells of porcine.

54. The assay of claim 45, wherein said assaying step further comprises:
adding an substrate to the label to the wells; and
detecting for a color reaction.

55. A method for determining the adherence characteristics of *Mycoplasma hyopneumoniae*, comprising:
providing a microtiter plate having stably bound to the wells substantially purified cilia containing receptors for *Mycoplasma hyopneumoniae*;
adding a suspension of *Mycoplasma hyopneumoniae* cells to the wells;
incubating the wells at about 37°C for a time sufficient to facilitate binding of the *Mycoplasma hyopneumoniae* cells to the receptors;
adding a wash solution to remove non-adherent *Mycoplasma hyopneumoniae* cells;
adding antibody to *Mycoplasma hyopneumoniae* to the wells;
incubating the wells at about 37°C for a time sufficient to facilitate binding of the antibody to the Mycoplasma hyopneumoniae cells;

adding a wash solution to remove unbound antibody;

adding labelled anti-immunoglobulin antibody to the wells;

incubating the wells at 37°C for a time sufficient to facilitate binding of the labelled anti-immunoglobulin antibody to the antibody;

adding a wash solution to remove unbound labelled anti-immunoglobulin antibody; and

assaying for the presence of label, thereby determining adherent *Mycoplasma hyopneumoniae* cells.

56. The assay of claim 55, wherein said assaying step further comprises:

adding an substrate to the label to the wells; and

detecting for a color reaction.

57. A method of screening for a high-adherent clone of *Mycoplasma hyopneumoniae* comprising:

immobilizing substantially purified cilia containing receptors for *Mycoplasma hyopneumoniae* to the wells of a microtiter plate;

reacting the bound cilia with a *Mycoplasma hyopneumoniae* suspension cultured from a single clone;

detecting for the presence of clones bound to the cilia.

58. The method of claim 57, wherein said detecting step further comprises:

reacting the adhered *Mycoplasma hyopneumoniae* clones with an antibody to *Mycoplasma hyopneumoniae*;

reacting labelled anti-immunoglobulin antibody with the bound antibody; and

assaying for the presence of label, thereby determining the presence of high-adherent clones of Mycoplasma hyopneumoniae.

59. The assay of claim 58, wherein said assaying step further comprises:

adding an substrate to the label to the wells; and

detecting for a color reaction.

60. The assay of claim 59, wherein said assaying step further comprises the step of:

comparing the color reaction to a standard in order to quantitate the adherence characteristics of the clone.

61. A quantitative assay for use in the production of a *Mycoplasma hyopneumoniae* adhesin-based vaccine:

immobilizing substantially purified cilia containing receptors for *Mycoplasma hyopneumoniae* to the wells of a microtiter plate;

reacting the bound cilia with *Mycoplasma hyopneumoniae* vaccine antigen;

detecting for the presence of antigen bound to the cilia.

62. The assay of claim 61, wherein said detecting step comprises:

reacting the bound antigen with an antibody to *Mycoplasma hyopneumoniae*;

reacting labelled anti-immunoglobulin antibody with the bound antibody; and

assaying for the presence of label, thereby detecting the presence of antigen.

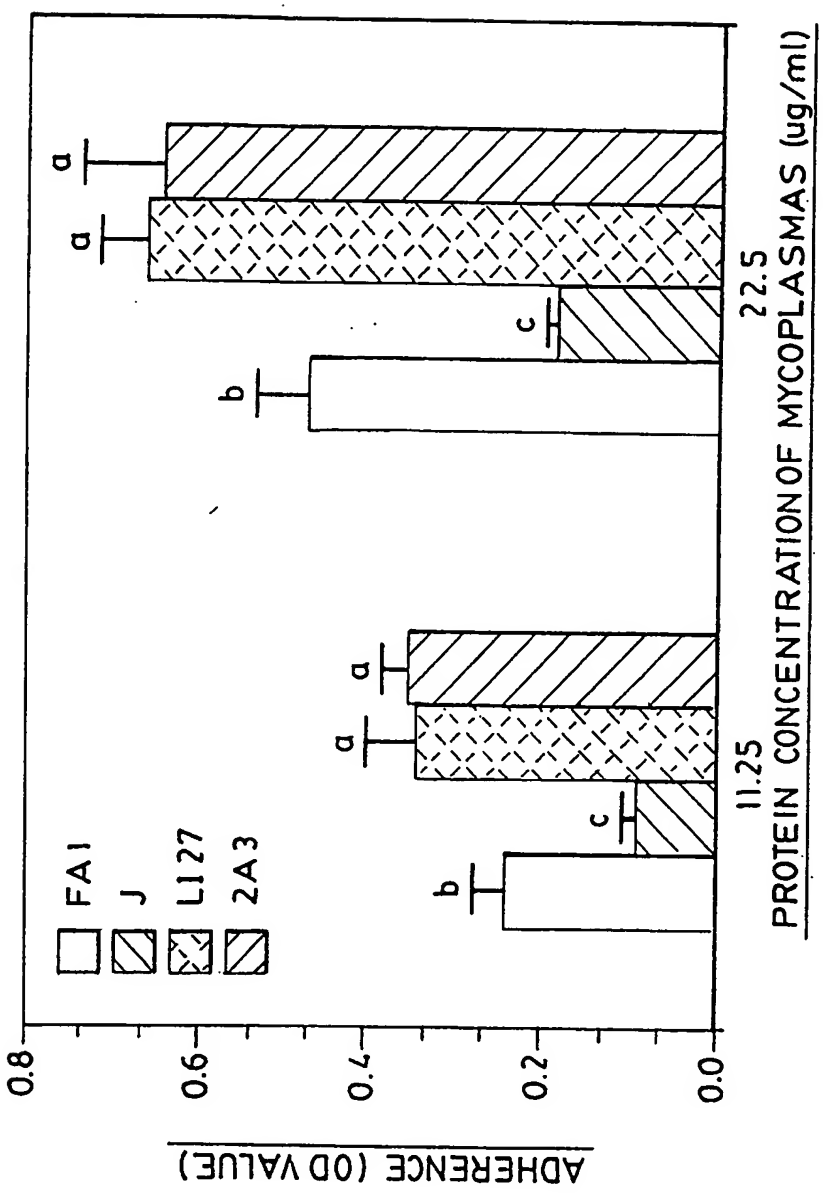
ADHERENCE (OD VALUE)
0.0
0.2
0.4
0.6
0.8
FA1
J
L127
2A3
a
a
b
c
11.25
a
a
b
c
22.5
PROTEIN CONCENTRATION OF MYCOPLASMAS (ug/ml)

FIG.1

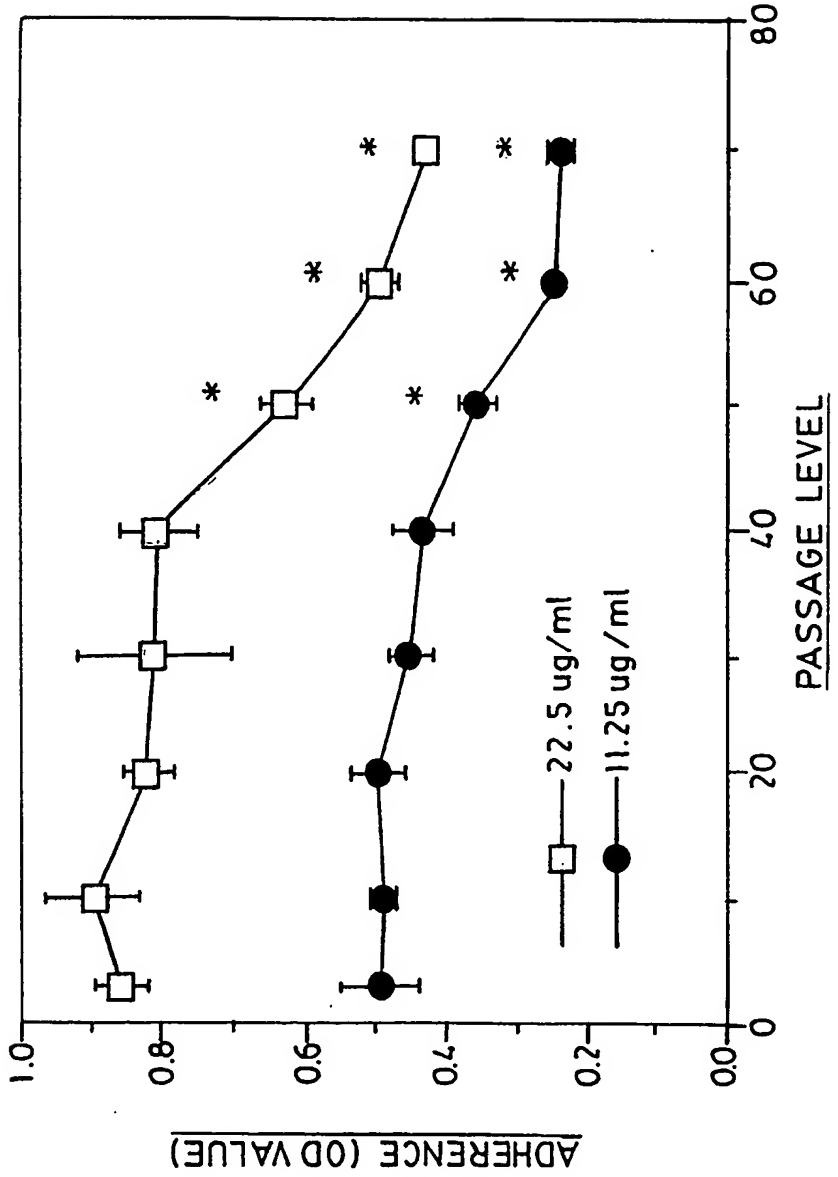
ADHERENCE (OD VALUE)
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
PASSAGE LEVEL
22.5 ug/ml
11.25 ug/ml

FIG.2

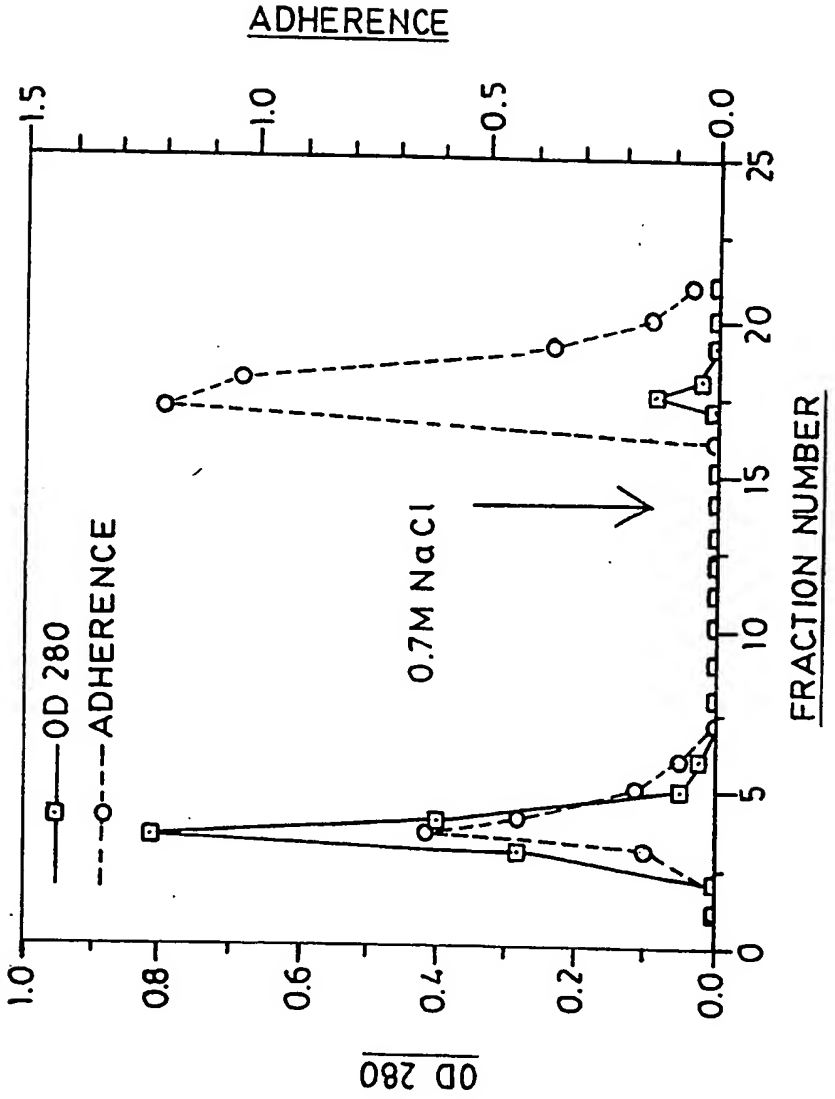
ADHERENCE
1.5
1.0
0.5
0.0
25
20
15
10
5
0
FRACTION NUMBER
0.7M NaCl
OD 280
ADHERENCE
1.0
0.8
0.6
0.4
0.2
0.0
OD 280

FIG. 3

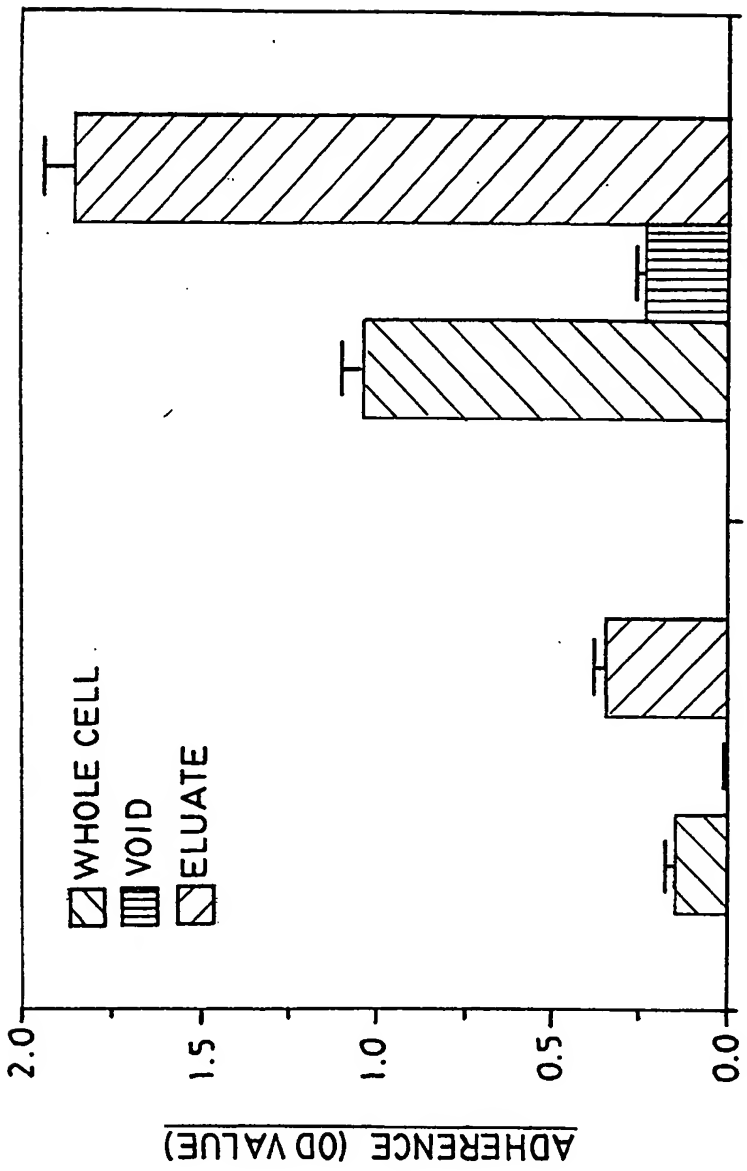
ADHERENCE (OD VALUE)
2.0
1.5
1.0
0.5
0.0
WHOLE CELL
VOID
ELUATE
1
5
PROTEIN CONCENTRATION (ug/ml)

FIG. 4

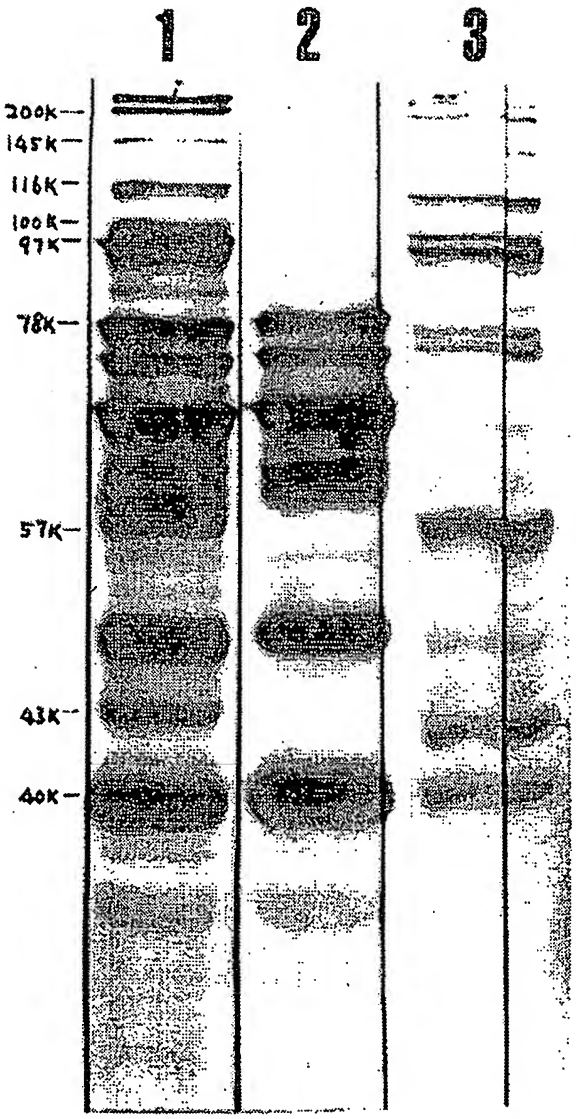

FIG. 5

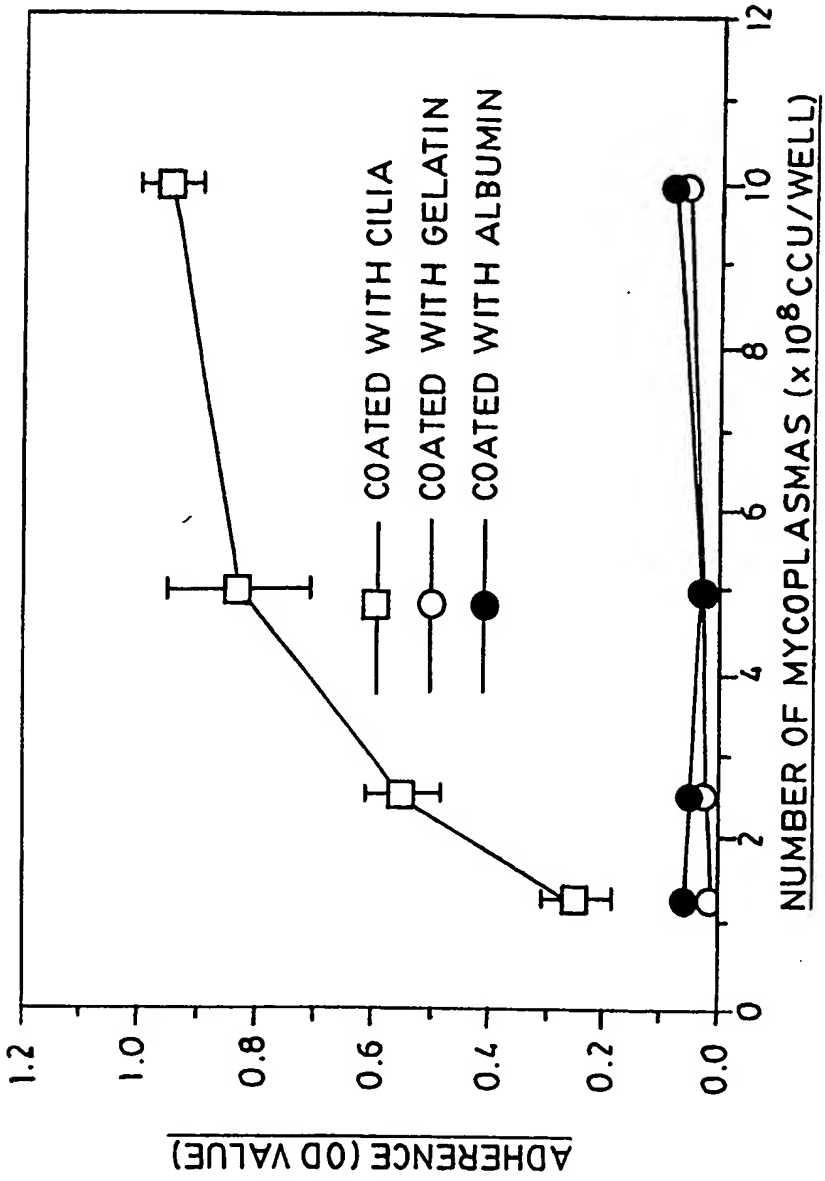
ADHERENCE (OD VALUE)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
12
NUMBER OF MYCOPLASMAS (x 10^8 CCU/WELL)
COATED WITH CILIA
COATED WITH GELATIN
COATED WITH ALBUMIN

FIG.6

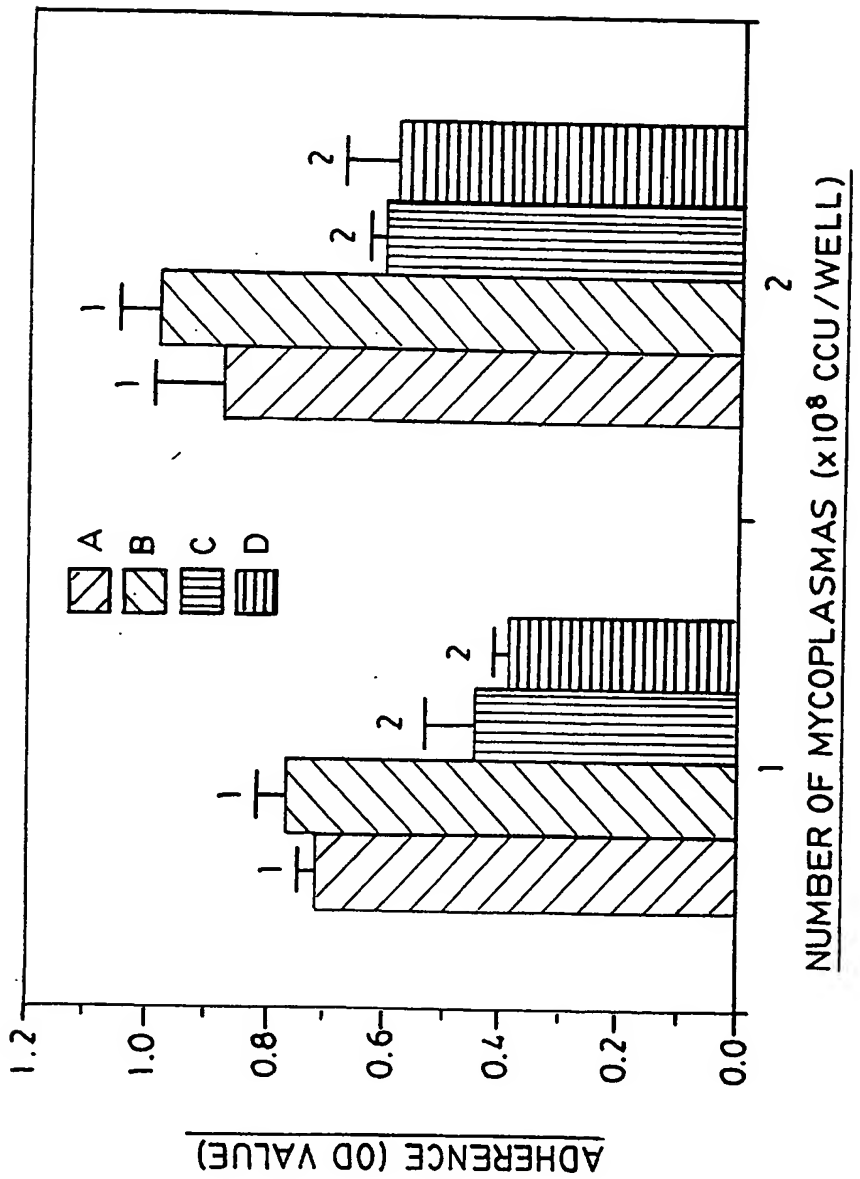
ADHERENCE (OD VALUE)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
A
B
C
D
1
2
NUMBER OF MYCOPLASMAS (x10⁸ CCU/WELL)

FIG. 7

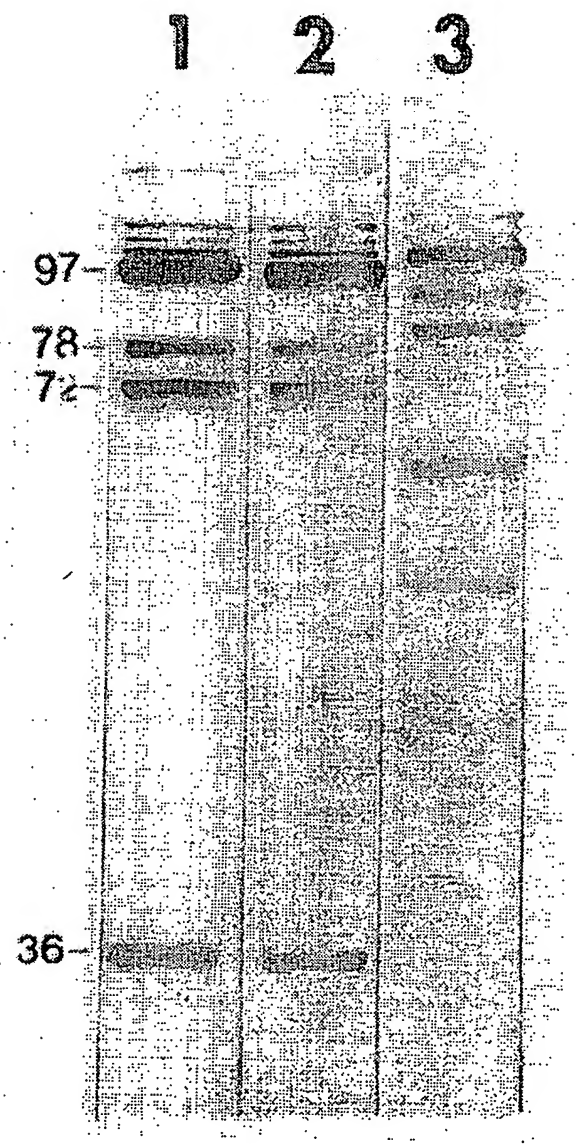

FIG.8

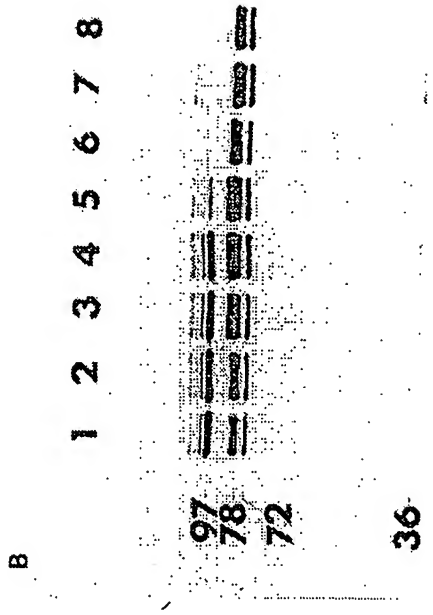

FIG.9B

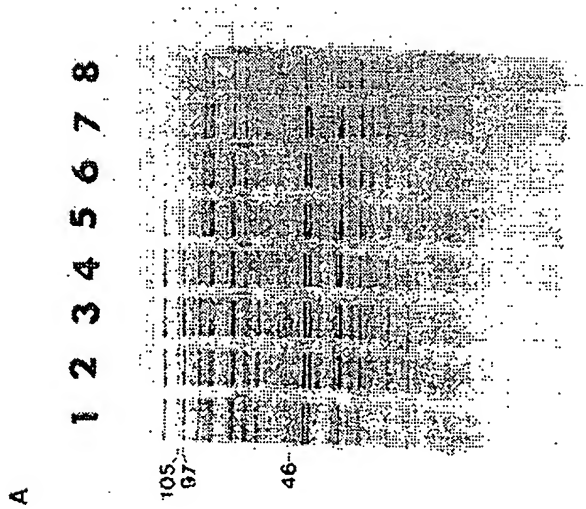

FIG.9A

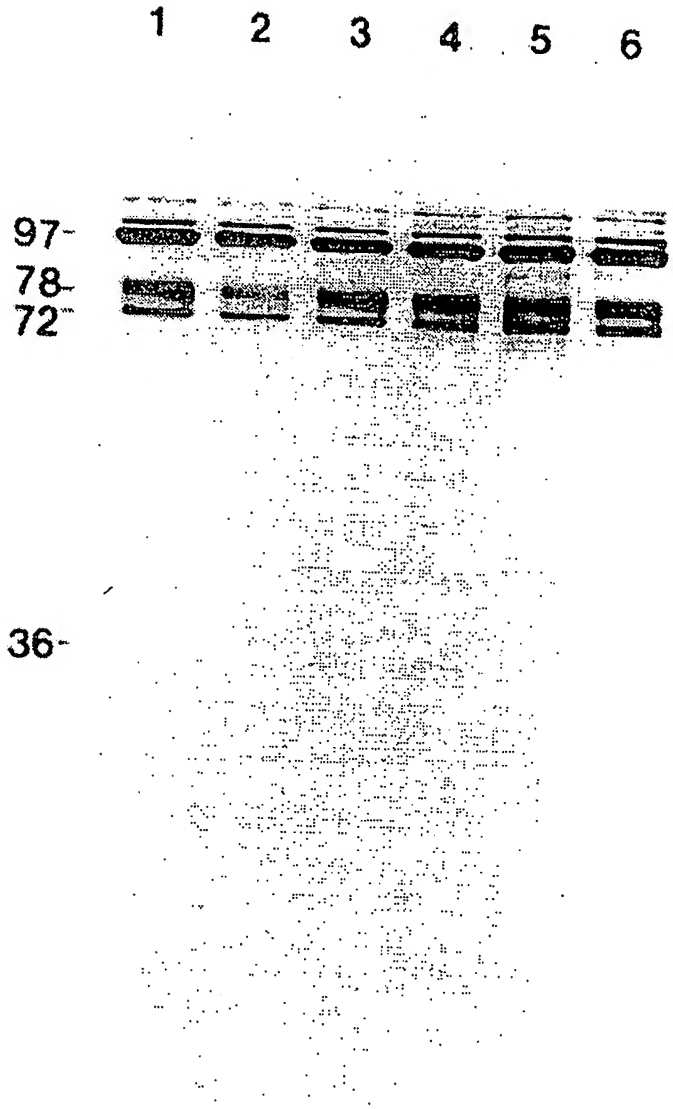

FIG.10

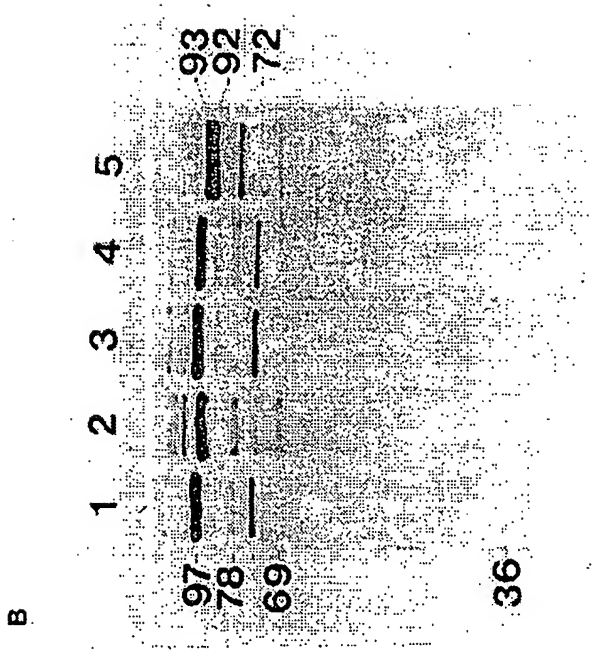

FIG.11B

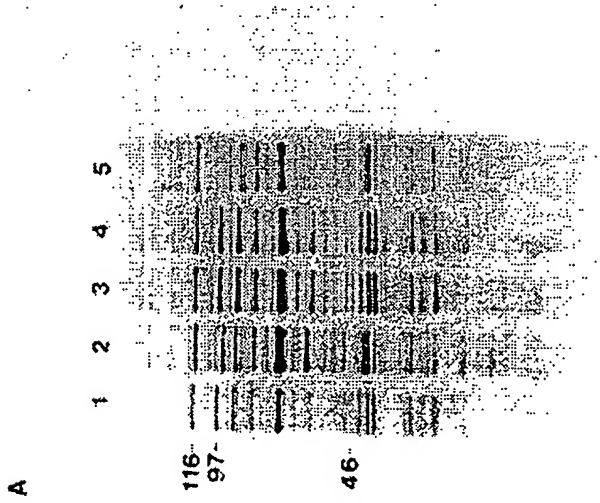

FIG.11A

FIG.12B

FIG.12A

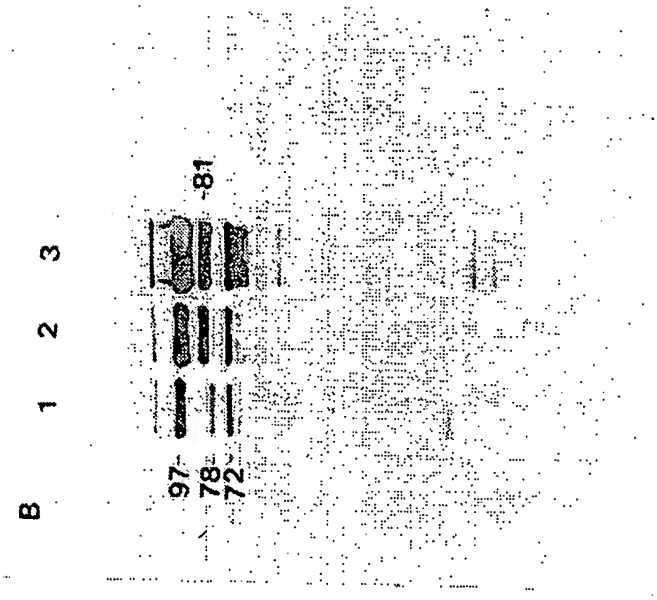

FIG.13B

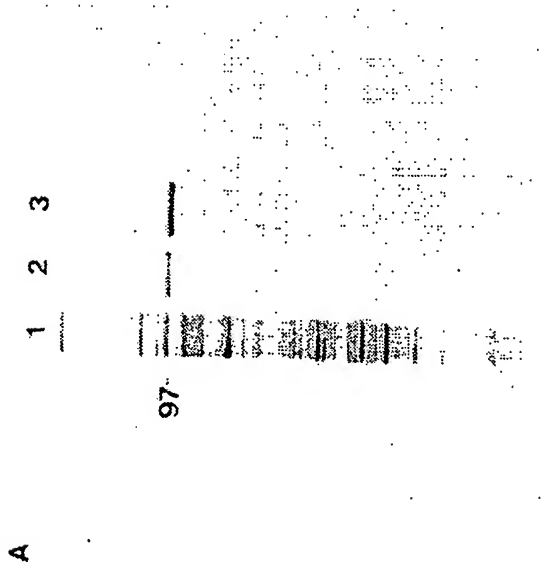

FIG.13A

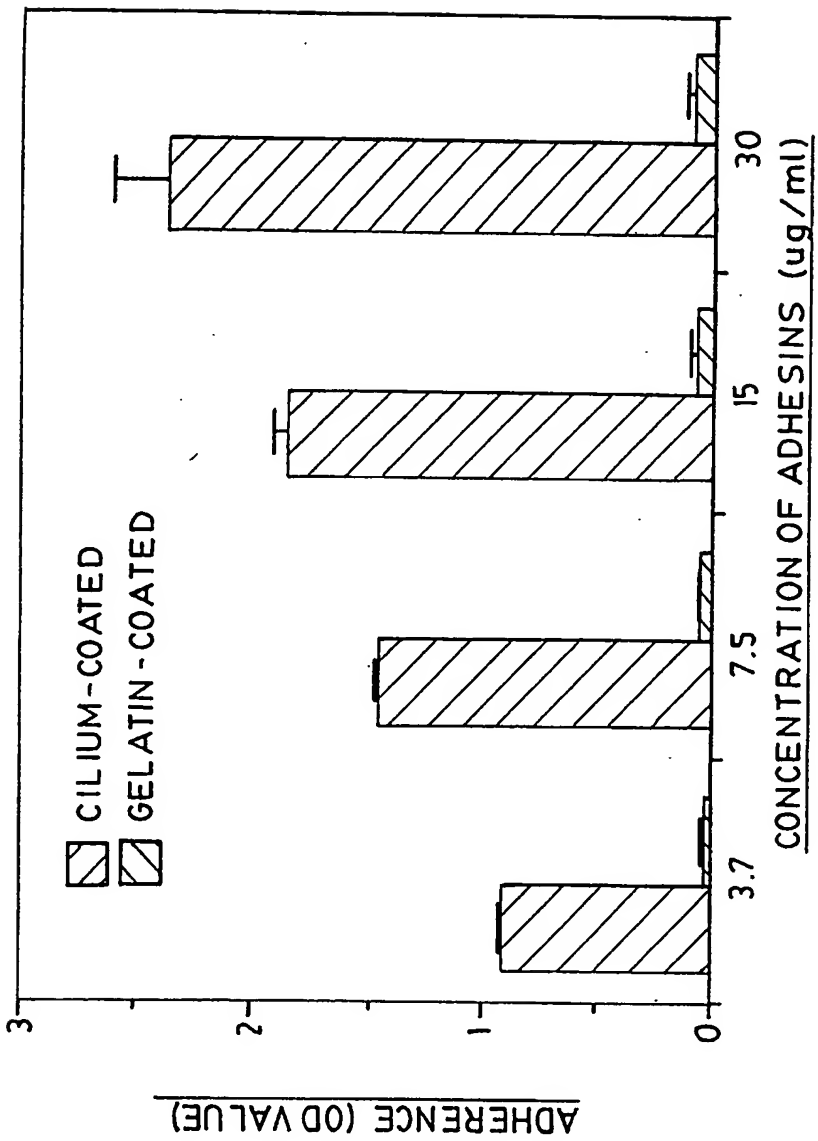
ADHERENCE (OD VALUE)
0
1
2
3
3.7
7.5
15
30
CONCENTRATION OF ADHESINS (ug/ml)
CILIUM-COATED
GELATIN-COATED

FIG. 14

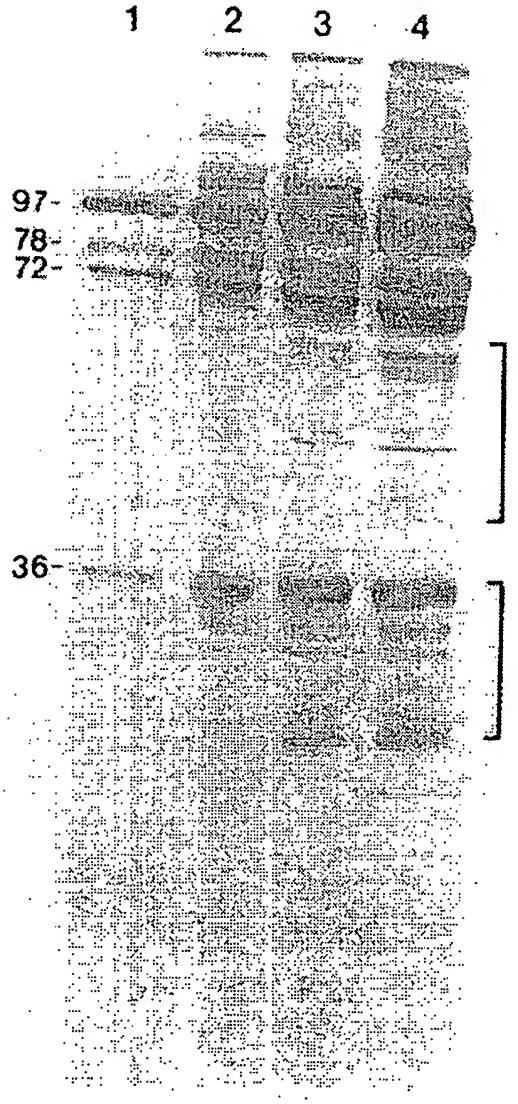

FIG. 15

16/16

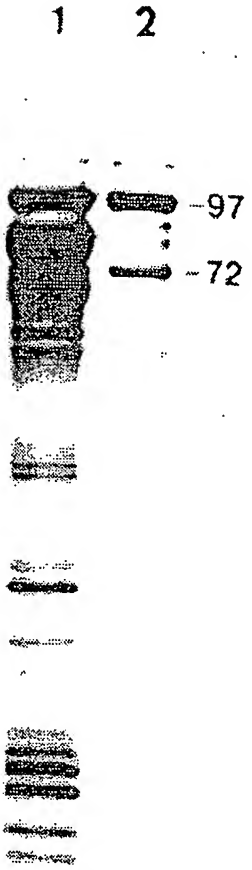

FIG. 16

INTERNATIONAL SEARCH REPORT

Internat ul Application No
PCT/uS 94/11320

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/30 A61K39/02 C07K16/12 A61K39/40 G01N33/53
C12P21/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
O,X	ABSTRACTS OF THE AMERICAN SOCIETY FOR MICROBIOLOGY,93RD GENERAL MEETING, May 1993, ATLANTA,GEORGIA,USA page 162 ZHANG ET AL 'PARTIAL CHARACTERIZATION OF MYCLOPLASMA HYOPNEUMONIAE ADHESINS'	1-7,9, 11-18, 29-31, 33-42, 44,53, 57,61
Y	see abstract G-1	19-21, 24-28, 32,43,62
	--- -/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 February 1995	16.02.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Sitch, W

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INTERNATIONAL SEARCH REPORT

Internal Application No
PCT/US 94/11320

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
O,X	ABSTRACTS OF THE AMERICAN SOCIETY FOR MICROBIOLGY,92ND GENERAL MEETING, May 1992, NEW ORLEANS,LOUISIANA,USA page 164 ZHANG ET AL 'CHARACTERIZATION OF RECEPTORS FOR MYCOPLASMA HYOPNEUMONIAE ADHERENCE TO SWINE RESPIRATORY EPITHELIUM' cited in the application	44-60
Y	see abstract G-30 ---	62
X	AMERICAN JOURNAL OF VETERINARY RESEARCH, vol.54, no.8, August 1993, CHICAGO,ILL.,USA pages 1262 - 1269 ZIELINSKI ET AL 'ADHERENCE OF MYCLOPLASMA HYOPNEUMONIAE TO PORCINE CILIATED RESPIRATORY TRACT CELLS' cited in the application	44-60
Y	see the whole document ---	62
Y	EP,A,0 359 919 (ML TECHNOLOGY VENTURES,L.P.) 28 March 1990 see page 2, line 1 - page 6, line 35 ---	19-21, 24-28, 32,43,62
O,A	IOM LETTERS,VOLUME.2;PROGRAM AND ABSTRACTS OF THE 9TH INTERNATIONAL CONGRESS OF THE INTERNATIONAL ORGANIZATION FOR MYCOPLASMOLOGY, August 1992, AMES,IOWA,USA page 321 YOUNG ET AL 'ANALYSIS OF VILRULENCE-ASSOCIATED ANTIGENS OF MYCOPLASMA HYOPNEUMONIAE' cited in the application see abstract ---	
A	THE JOURNAL OF EXPERIMENTAL MEDICINE, vol.168, no.1, 1 July 1988, NEW YORK,USA pages 267 - 277 TUOMANEN ET AL 'RECEPTOR ANALOGS AND MONOCLONAL ANTIBODIES THAT INHIBIT ADHERENCE OF BORDETELLA PERTUSSIS TO HUMAN CILIATED RESPIRATORY EPITHELIAL CELLS' cited in the application see page 275,summary ---	
A	AMERICAN JOURNAL OF VETERINARY RESEARCH, vol.48, no.4, April 1987, CHICAGO.ILL.,USA pages 651 - 656 YOUNG ET AL 'ASSESSMENT OF ANTIBODY RESPONSE OF SWINE INFECTED WITH MYCOPLASMA HYOPNEUMONIAE BY IMMUNOBLOTTING' cited in the application see page 651,summary,pages 654-656,'discussion' ---	

-/--

3

INTERNATIONAL SEARCH REPORT

Internal Application No
PCT/US 94/11320

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	INFECTION AND IMMUNITY, vol.62, no.5, May 1994, WASHINGTON D.C.,USA pages 1616 - 1622 ZHANG ET AL 'MICROTITER PLATE ADHERENCE ASSAY AND RECEPTOR ANALOGS FOR MYCLOPLASMA HYOPNEUMONIAE'	44-60
P,Y	see the whole document -----	62

3

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

:national application No.
PCT/US94/11320

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 12, 13, 17, 18, 24-28 are directed to a method of treatment of the animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

.ormation on patent family members

Intern· ·al Application No
PCT/US 94/11320

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0359919	28-03-90	US-A-	5240706	31-08-93
		JP-A-	2291271	03-12-90

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